Investigating the role of nutrient-sensing pathways and senescence in stem cell competition and early mammalian development

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Declaration of originality

I certify that this thesis, and the research to which it refers, are the product of my own work apart from where otherwise stated. Any ideas or quotations from the work of other people are fully acknowledged in accordance with the standard referencing practices of the discipline.

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Abstract

Cell competition is a quality control mechanism that eliminates cells which are viable but less fit than surrounding cells. The outcome of cell competition is enhanced organismal fitness as the process prevents the propagation of cells which are oncogenic, mispatterned, or otherwise sub-optimal. Cell competition research has largely been performed in flies, although recently it was found that cells in the early mammalian embryo are sensitive to competitive interactions. However, the underlying mechanisms driving the elimination of less fit cells in this system remain largely unknown. Here, we interrogate competition mechanisms in the early mammalian embryo. First, we postulate that senescence may have a role in mediating the out-competition of defective cells. While our evidence does not provide support for this framework, we observed an upregulation of senescence-associated phenotypes in the early post-implantation epiblast. Second, we find that the confrontation of cells with different fitness levels leads to a repression of mTOR signalling in the less-fit cell type. This repression is sufficient to trigger apoptosis, and hyperactivation of the mTOR pathway rescues loser cell elimination and allows less-fit tetraploid cells to contribute to later developmental stages. Finally, we find that less-fit cell types are marked by elevated levels of p53, and loss of p53 prevents both loser cell elimination and mTOR pathway repression. Furthermore, mutations affecting p53/mTOR activity increase the competitive ability of cells within the embryo and cause tissue overgrowths that prevent proper morphogenesis and patterning. Our results shed light on the mechanisms of cell competition in development but also allude to a role for cell competition in the elimination of damaged or nutrient-stressed cells prior to gastrulation.

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Abbreviations

ATP	Adenosine triphosphate
APC	Adenomatous polyposis coli
BMP	Bone morphogenetic protein
BMPR1A	BMP receptor 1a
BSA	Bovine serum albumen
CRISPR	Clustered regularly interspersed short palindromic repeats
DMEM	Dulbecco's modified Eagle's serum
DNA	Deoxyribonucleic acid
Dpp	Decapentaplegic
EDAC	Epithelial defence against cancer
ESC	Embryonic stem cell
ExE	Extraembryonic ectoderm
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Fig.	Figure
GAP	GTPase activating protein
GDI	GTP dissociation inhibitor
GEF	GTP exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HSC	Hematopoietic stem cell
IF	Immunofluorescence
IPA	Ingenuity Pathway Analysis
ISC	Intestinal stem cell
IVF	*In vitro* fertilisation
JNK	c-Jun N-terminal kinase
kda	Kilodalton
LIF	Leukaemia inhibitory factor
MSCV	Murine stem cell virus
mTOR	mechanistic (formerly mammalian) target of rapamycin
mTORC1	mechanistic (formerly mammalian) target of rapamycin complex 1
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative PCR
RNA	Ribonucleic acid
S6	Ribosomal protein S6
S6K	S6 kinase
SAβG	Senescence-associated β-galactosidase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
shRNA	short hairpin RNA
siRNA	short interfering RNA
TFEB	Transcription factor EB
TSC	Tuberous sclerosis complex
VE	Visceral endoderm
wt	Wild-type

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Chapter 1: Introduction

1.1. Cell competition

1.1.1. Darwin's theory of evolution

In 1859, Darwin published *On the Origin of Species by Means of Natural Selection* and transformed our way of thinking about life on Earth. The publication provided support for the developing idea that the existence of all life is determined by the inheritance of traits which affect the ability of organisms to survive, compete, and reproduce (Darwin, 1859). Not long after, in 1881, zoologist Wilhelm Roux proposed that cells in a multicellular organism are also subject to the same evolutionary pressures as animals in the wild, and that this pressure enhances organismal fitness by selecting against weaker mutations (Roux, 1881). Darwin proposed that natural selection in the animal kingdom depends on three principles factors: that variation exists among individuals; that this variation is genetically encoded and heritable; and that variation results in differential survival capabilities that lead to selection of the strongest phenotypes. Cells within tissues share these same hallmarks: single-cell sequencing has revealed that a wide degree of cell heterogeneity occurs within tissues (Altschuler and Wu, 2010); certain mutations can increase or decrease the ability of cells to thrive in different environments; and evidence collected over the past 50 years has revealed that competition occurs between cells in insects, fish and mammals.

1.1.2. Cell competition

Cell competition describes a cellular interaction which results in the elimination of a viable but less fit cell specifically when in the presence of a more fit cell. This elimination can occur through apoptosis, activation of cellular senescence, cell extrusion or cell differentiation. The eliminated less-fit cells are named the "loser" cells, while the persisting more-fit cells are labelled the "winner" cells. Experimentally the first evidence of the context-dependent elimination of cells was first observed in the 1970s between cells of the developing imaginal wing disc in flies (Morata and Ripoll, 1975). Since then, the field has expanded rapidly as more studies were carried out over the late 20th century in *Drosophila*, and, in the last decade, in mammals. Studies were largely descriptive of cell competition until recently, where its physiological roles, particularly in relation to tumourigenesis, have been examined in greater detail.

1.1.3. Different processes of cell competition

At present, cell competition is used as an umbrella term for several different processes (Fig. 1.1). Since there are significant gaps in our knowledge as to the underlying mechanisms of these different forms of cell competition, it is difficult to determine the degree to which they are separate entities with resembling features, or all the same process with different phenotypes. Gaining understanding for the mechanisms responsible for competition will help clarify this in future. For this thesis however, I have separated five different sub-categories of the process. Mostly I will discuss the largest field of cell competition, "classical" cell competition, which in this thesis describes the elimination of less fit cells through apoptosis or senescence. At the end of the introduction I will briefly outline the significant findings of the other fields: the neurotrophic theory; epithelial defence against cancer (EDAC); stem cell neutral drift; and cancer-host competition.

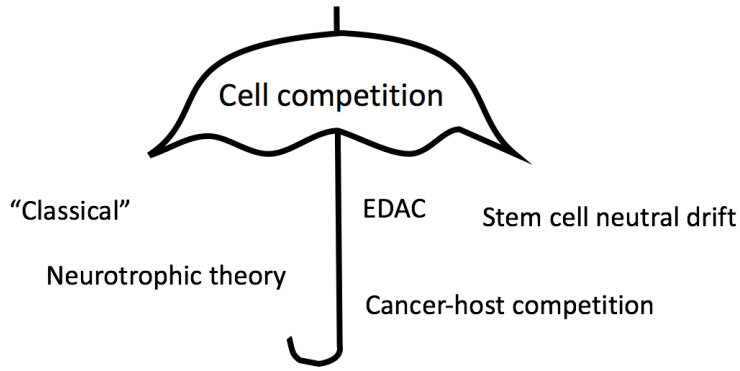


Figure 1.1: Umbrella of cell competition.

Cell competition currently encapsulates several different subgroups including: "classical" competition; the neurotrophic theory; epithelial defence against cancer (EDAC); stem cell neutral drift; and cancer-host competition.

1.2. Cell competition in development

The majority of "classical" cell competition research has been performed in developing tissues. Here I will outline the triggers and occurrence of competition in developmental tissues of *Drosophila* and mammals.

1.2.1. History of cell competition in developing *Drosophila* tissues

The first losers: Minute *mutants*

Cell competition was originally characterised in *Drosophila*. The first studies were performed through mosaic expression of ribosomal subunit-encoding *Minute* genes in the imaginal wing

disc (Morata and Ripoll, 1975). Flies with heterozygous *Minute* mutations are viable and fertile, albeit with mild phenotypic abnormalities including shortened bristles and slowed development. However, when clones of *Minute*$^{+/-}$ cells are formed in a wild-type background, these clones are eliminated by apoptosis, and are not recovered in the adult fly wing (Morata and Ripoll, 1975; Moreno et al., 2002; Simpson, 1979). The process is phenotypically silent, as wild-type cells undergo compensatory proliferation to offset the loss of cells from the tissue.

Many mutations trigger cell competition

Subsequent research led to the discovery of numerous *Drosophila* mutations which trigger cell elimination specifically in a competitive environment. As with *Minute* mutations, reducing cell growth and proliferation through depletion of dMyc or Ras results in the apoptotic elimination of cells specifically when in the presence of wild-type cells (Johnston et al., 1999; Moreno and Basler, 2004; Prober and Edgar, 2000). Mutations which affect signalling pathways also act as losers in competitive scenarios. These include mutations in the insulin (Böhni et al., 1999; Verdu et al., 1999), BMP (Burke and Basler, 1996), Wnt (Vincent et al., 2011), JAK/STAT (Rodrigues et al., 2012) and Hippo (Neto-Silva et al., 2010; Tyler et al., 2007) signalling pathways. Furthermore, clones of mispatterned cells undergo apoptosis in a seemingly non-autonomous fashion (Milán et al., 2002). Finally, cells with disrupted polarity due to mutations in *scribble* (*scrib*), *lethal giant larvae* (*lgl*) and *disc large* (*dlg*) which cause disordered, neoplastic overgrowth of cells in a homotypic environment, are eliminated when surrounded by wild-type cells (Agrawal et al., 1995; Brumby and Richardson, 2003; Menéndez et al., 2010; Yamamoto et al., 2017). These *Drosophila* studies have been performed both in the developing imaginal wing disc and the developing eye (Brumby and Richardson, 2003; Menéndez et al., 2010; Tyler et al., 2007).

Super-competition

Mutations have also been described which enhance cell competitiveness and induce the elimination of wild-type cells. This ‘super-competition’ was first demonstrated through mosaic overexpression of oncogene dMyc in imaginal wing discs (de la Cova et al., 2004; Moreno and Basler, 2004). Increasing Myc in clones of cells results in the apoptotic elimination of surrounding wild-type cells. Since then, cells carrying mutations in the Hippo pathway have been shown to act as super-competitors by activating Myc (Ziosi et al., 2010), while Myc-

independent super-competition has also been demonstrated through hyper-activation of the Wingless and JAK/STAT signalling pathways (Rodrigues et al., 2012; Vincent et al., 2011).

1.2.2. Cell competition in mammalian development

There are now a number of studies which demonstrate that cell competition also occurs during mammalian development. Like studies in *Drosophila*, mutations in ribosomal subunits can trigger cell competition in mouse. For example, although mice with heterozygous mutations in ribosomal gene *Rpl24* survive until adulthood with mild phenotypic abnormalities (Oliver et al., 2004), when mixed with wild-type cells in mouse chimeras, $Rpl24^{+/-}$ cells contribute poorly to adult mice. Similarly, cells with elevated levels of p53 (caused by haplo-insufficiency of p53 inhibitors *Mdm2/4*) have a selective disadvantage in a wild-type background, although non-chimeric $Mdm2/4^{+/-}$ mice are viable (Zhang et al., 2017). Super-competition has also been shown to occur during development: cells lacking p53 act as 'cooperation cheaters' which outcompete wild-type cells (Dejosez et al., 2013). It is not clear from these studies whether cell competition results from reduced growth or increased death of losers, but the studies demonstrate that cell competition is conserved from flies to mammals.

Cell competition during gastrulation

Although the above studies demonstrate that certain viable genotypes can confer upon cells a selective disadvantage in a wild-type background early on in life, it is unclear whether the loser cells are faced with continual competitive pressure throughout development or whether they are eliminated at a certain developmental stage. Recent evidence has suggested that cell competition is particularly acute at a specific stage in embryogenesis: at the onset of gastrulation, where cells differentiate to form the three germ layers.

After fertilisation in the mouse, pluripotent cells in the developing blastocyst divide synchronously and slowly (Kojima et al., 2014). At embryonic day (E) 4.5, the embryo implants into the uterine wall and an enormous increase in cell proliferation occurs (Fig. 1.2; Kojima et al., 2014; Solter et al., 1971). In two days, cell numbers in the portion of the embryo which gives rise to the embryo proper, the epiblast, rise from 120 cells (at E5.5) to 8060 cells (at E7.5). This massive increase in cell proliferation occurs to generate cells for the initiation of gastrulation at E6.5 (Solter et al., 1971), and it is conceivable that the process results in the emergence of cells with mutations as a result of DNA replication errors. Accordingly, the onset

of gastrulation is associated with hyper-sensitivity to DNA damage (Fig. 1.2; Heyer et al., 2000; Laurent and Blasi, 2015) which is thought to act as a stringent quality control mechanism to ensure the removal of cells which have become damaged prior to their clonal expansion. Similarly, recent data suggest that cell competition acts at this stage to work alongside the hypersensitivity to DNA damage as a mechanism to remove cells with reduced *relative* fitness (Fig. 1.2). In this way, cell competition in the early post-implantation epiblast is thought to be a mechanism which optimises embryonic fitness.

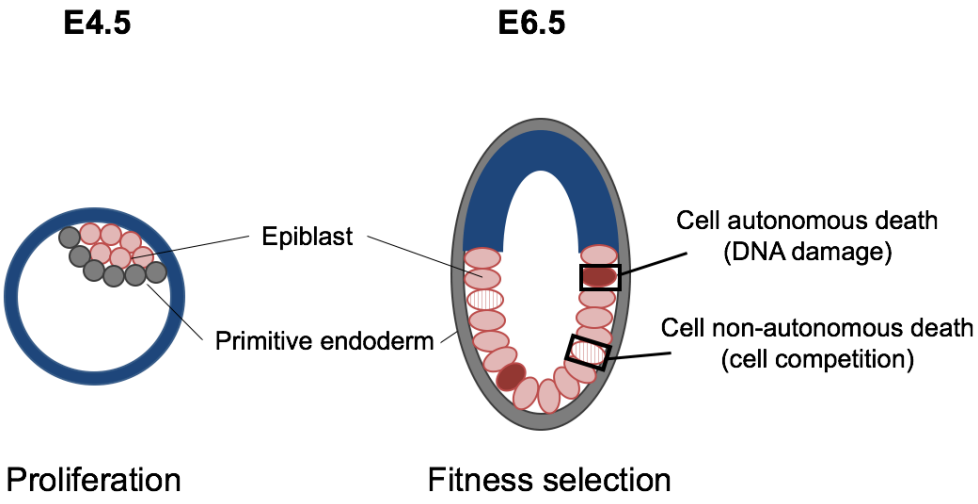


Figure 1.2: Fitness selection during early embryogenesis.
Massive proliferation occurs in the epiblast cells (pink) of the embryo between E4.5 and E6.5. Fitness selection is mediated by hypersensitivity to DNA damage, which initiates the removal of damaged or unfit cells in an autonomous manner; and cell competition, which initiates the removal of damaged or less fit cells in a non-autonomous manner.

Data from two studies support this hypothesis. In the first, a range of viable but suboptimal mouse embryonic stem cells (ESCs; tetraploid cells, cells with defective BMP signalling, and cells with defective autophagy) are shown to be eliminated *in vitro* in a culture assay which mimics the initial differentiation stages of embryonic gastrulation (Sancho et al., 2013). Importantly, these cells are viable when cultured alone or with other less-fit cells. The study also provides evidence that cell competition occurs *in vivo*: while it was known previously that tetraploid embryos can survive until late gestation or birth (Kaufman, 1991; Snow, 1975), when tetraploid cells are injected into diploid embryos they undergo apoptosis at ~E6.5 (Sancho et al,. 2013). This fits with evidence from other studies indicating that karytypically abnormal

cells are eliminated during embryogenesis in both humans and mice (Bolton et al., 2016; Eakin et al., 2005; Greco et al., 2015; Sancho et al., 2013).

In the second study, relative levels of Myc are shown, like in *Drosophila*, to dictate competitive interactions (Claveria et al., 2013). Generation of a mouse embryo with mosaic over-expression of *cMyc* results in the apoptotic elimination of wild-type cells with normal, but relatively lower, *cMyc* levels (Claveria et al., 2013). Post-gastrulation, high proportions of high-Myc cells are observed across various tissues, indicating that cell competition occurring in early embryogenesis dictates which cells give rise to future organ development. Furthermore, endogenous levels of Myc in wild-type embryos have been shown to be highly heterogeneous and can be correlated with death, with low-Myc cells being the most likely to die (Claveria et al., 2013). This last finding demonstrates that cell competition could be an active endogenous mechanism during gastrulation.

Cell competition at later stages of development

Cell competition also occurs at later stages in embryonic development. During heart development, cardiomyocyte progenitors overexpressing *cMyc* have a competitive advantage over wild-type cells, which are depleted from the developing tissue through apoptosis (Villa del Campo et al., 2014). Although the occurrence of endogenous cell competition in this tissue is not known, this study reveals that developing cardiomyocytes are sensitive to Myc-induced competition.

1.3. Cell competition in adult tissues

Cell competition is not confined to developing tissues: the process has been studied in a wide variety of adult tissues in mouse, *Drosophila*, and various mammalian cell lines. Here I will outline the triggers and occurrence of competition in adult tissues of *Drosophila* and mammals.

1.3.1. Adult stem cell niches

Cell competition has been demonstrated in various adult stem cell niches in *Drosophila* and mice. Homeostasis of stem cell number is maintained through stem cell renewal or differentiation, and during stem cell competition, loser cells are eliminated from the functional stem cell pool through differentiation and subsequent displacement from the niche.

Cell competition in this setting occurs both as a stochastic process, termed neutral stem cell drift (to be discussed in the section *Stem cell neutral drift*) and as a biased one, termed non-neutral cell competition. Non-neutral competition occurs when one cell has a self-renewing competitive advantage over other cells in the niche. In this scenario, competition is induced by cancer-associated mutations (Vermeulen et al., 2013) and the confrontation of cells with different levels of proliferation (Snippert et al., 2013), differentiation ability (Jin et al., 2008), JAK/STAT signalling (Issigonis et al., 2009) or cell adhesion properties (Jin et al., 2008; Issigonis et al., 2009; Alcolea et al., 2014). Furthermore, some evidence also exists for Myc-dependent competition in the *Drosophila* ovary (Rhiner et al., 2009). In these studies it has been proposed that cell competition is used to balance the ratio of different cell types which co-opt the stem cell niche, although other work has provided evidence to show that competition can also occur to eliminate defective cells. In the adult *Drosophila* posterior midgut, *Minute*$^{+/-}$ intestinal stem cells (ISCs) are effectively eliminated by their wild-type counterparts (Kolahgar et al., 2015). The authors show through the use of mathematical modelling and analysis of ISC number per clone over time that the elimination of *Minute*$^{+/-}$ cells is compensated for by an increase in both the proliferation and self-renewal of wild-type ISCs. Furthermore, the surrounding tissue recovery is mediated by JNK-dependent JAK/STAT activation. Collectively, these studies demonstrate that cell competition occurs in the adult stem cell niche, and that cell competition in this setting bears similarity with developmental cell competition, such as the involvement of Myc and the JAK/STAT pathway.

1.3.2. Liver and heart

Cell competition can also occur in the adult liver and heart in rats and mice, respectively. In rats, cell competition has been attributed to high levels of liver regeneration (Oertel et al., 2006). Transplantation of fetal liver stem/ progenitor cells into the livers of rats which have undergone a partial hepatectomy results in a high level of contribution from the donor cells after 6 months. This occurs not only as a result of high levels of proliferation of the donor cells, which differentiate into hepatocytes and function normally within the new tissue, but also to increased levels of death of the hepatocytes within the host tissue (Oertel et al., 2006). Although studies investigating the aptitude of such donor cells in populating diseased organs have not yet been performed, this study reveals that cell competition could be exploited in regenerative medicine. The hearts of adult mice are also sensitive to competition induced by *cMyc* differences (Villa del Campo et al., 2014). Using an inducible system to generate mosaic levels of *cMyc* in adult mice cardiomyocytes, the authors of this study revealed that high Myc cells

replace wild-type cells in an apoptosis-dependent process. In summary, these two studies demonstrate that the heart and liver are sensitive to cell competition, although further work is required to understand to what degree cell competition occurs as an endogenous process during homeostasis or following damage.

1.3.3. Hematopoietic tissue and immune system cells

Competition has been reported in hematopoietic stem and progenitor cells (HSC and HSPCs) as a mechanism to select for the least damaged cells (Bondar and Medzhitov, 2010). Through use of bone marrow repopulation experiments and mosaic mouse models, the authors of this study demonstrate that irradiated cells have a competitive disadvantage compared with untreated cells. This effect is mediated through p53: loss of one copy of p53 in the irradiated cells prevents their outcompetition. Furthermore, altering p53 levels is sufficient to induce competition, as cells carrying only one copy of p53 outcompete *Mdm2*$^{+/-}$ cells, which have elevated p53 levels. In this instance, less fit cells are eliminated through senescence rather than apoptosis. Cell competition also occurs between young and old T-cells in the thymus to ensure the replacement of older T-cells by their younger counterparts (Martins et al., 2014).

1.3.4. Post-mitotic tissues

Recently, cell competition has been described to occur in post-mitotic cells. Deletion of one copy of *Minute* or Lgl-binding protein *Mahjong* from cells in the *Drosophila* follicle results in cell apoptosis in mosaic tissues, but not homotypic ones (Tamori and Deng, 2013). An interesting difference of this study to others is that rather than inducing compensatory proliferation, death of loser cells activates compensatory cellular hypertrophy of surrounding cells. Cell competition interactions have also been described between post-mitotic cardiomyocytes in mice and intestinal enterocytes in *Drosophila* (Kolahgar et al., 2015; Villa del Campo et al., 2014).

1.3.5. Cell lines

Modelling cell competition in mammalian cell lines has given insight into several areas of cell competition research. First, use of Madin Darby Canine Kidney (MDCK) cells have revealed that mammalian cells carrying polarity mutations, like in the *Drosophila* imaginal wing disc, are outcompeted specifically when in the presence of wild-type cells (Norman et al., 2012). Secondly, they have revealed that cell competition is not restricted to epithelial tissues but also occurs in mesenchymal fibroblast cell cultures (Mamada et al., 2015). Finally, they have

revealed that less fit cellular subtypes arise stochastically in cultured cell lines and that these are subjected to out-competition (Penzo-Méndez et al., 2015).

1.4. Stages of competition

The cell interactions in cell competition can be split into three stages: a 'trigger' stage where a population of cells exhibit heterogeneous fitness levels, a 'fitness sensing' stage, and a cell elimination stage (Fig. 1.3). Here I will discuss what is known currently about each of these stages.

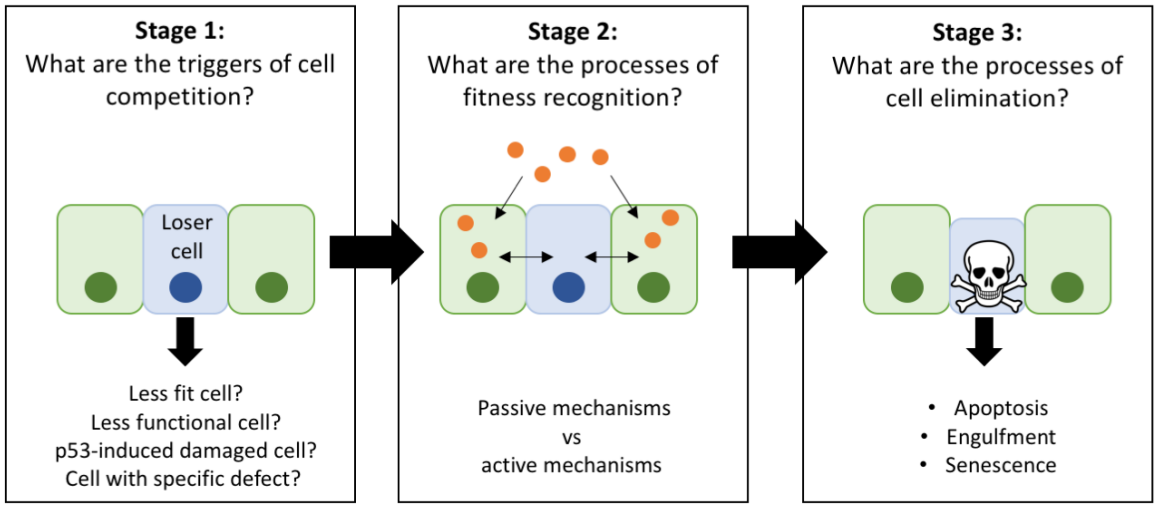


Figure 1.3: Stages of cell competition.

Competition can be split into three stages: a 'trigger' stage, where a tissue heterogeneity results in formation of loser cells; a recognition stage where loser cells are either sensed by neighbouring cells or possess a selective disadvantage; and an elimination stage where loser cells are removed from the tissue.

1.5. Stage 1: What are the triggers for cell competition?

Although cell competition has been shown in a variety of different tissues and systems, it is still unclear what exactly determines cellular fitness. Here I will outline four possible options for differences between cells that could trigger competition.

1.5.1. Evolutionary-based fitness

In an evolutionary sense, cell fitness would be defined as the ability of a cell to reproduce and pass on genetic material. In cells, mutations which increase cell proliferation could be thought to increase evolutionary fitness and therefore to trigger cell competition. Accordingly, many of the mutations which trigger competition affect proliferation rate. However, while

proliferation-enhancing mutations are often predictive of cell competition, there are exceptions. For example, cells with increased proliferation rates due to overexpression of cyclin D and CDK4 do not induce elimination of surrounding wild-type clones (de la Cova et al., 2004; Hafezi et al., 2012). Similarly, cells with mutations in polarity genes do not have a proliferation defect but are eliminated themselves by relatively slower-growing wild-type cells (Brumby and Richardson, 2003; Menéndez et al., 2010). Therefore, if cell fitness is simply the ability of a cell to proliferate in a given environment, this quality is not always sufficient to trigger cell competition.

1.5.2. Function-based fitness

An alternative possibility is that cell fitness, rather than being ability to thrive, is a measure of fulfilment of cell function. In this case, mutations triggering cell competition will be tissue-specific. Supporting this, Myc-induced competition occurs in *Drosophila* imaginal wing discs (de la Cova et al., 2004; Moreno and Basler, 2004), perhaps a tissue where high translation equals high fitness, but not in ovary stem cell niches, where adhesion and self-renewal capacity could be expected to mediate function-based fitness (Jin et al., 2008). Similarly, in post-mitotic epithelia, cell competition is not triggered in response to dMyc levels, possibly because cell function is not proliferation-dependent and so Myc cannot dictate fitness in this way. Instead, in stem cell niches, mutations affecting self-renewal and differentiation induce competition (Jin et al., 2008), although these same mutations would not be expected to confer a competitive advantage upon imaginal disc cells. Unlike the evolutionary-based fitness possibility, in this case cell fitness would not be read as a universal code across all tissues but would be related to function and therefore be tissue-specific.

1.5.3. Stress-driven triggers

In both mammals and flies, loser cells frequently carry hallmarks of cellular stress. This could suggest that cell competition occurs to remove stressed cells when in the presence of less-stressed cells.

In mammals, this could be mediated by p53, a tumour suppressor protein which is activated in response to cellular damage and drives apoptosis, growth arrest, and senescence, among other pathways (Vousden and Prives, 2009). In various mammalian competition models, p53 is required for the elimination of loser cells and upregulation of the protein alone is sufficient to trigger cell competition (Bondar and Medzhitov, 2010; Wagstaff et al., 2016; Zhang et al.,

2017). It is feasible that if p53 is not activated to the point where autonomous cell death is triggered, differences in p53 levels between cells could trigger the elimination of the damaged or stressed cell through cell competition. It remains to be resolved how certain mutations result in supercompetition through this model, although it is possible that supercompetitor mutations could mechanistically mimic a low damage, low-p53 cell status. Alternatively, p53-induced cell competition could represent just one competition subgroup.

Interestingly, p53 is not required for cell competition to occur in *Drosophila* (Kale et al., 2015), and has been described to instead aid winner cell survival in Myc-competition models (de la Cova et al., 2014). However, loser cells in fly models share stress signatures of elevated JNK and oxidative stress, and elevation of oxidative stress gene Nrf2 is sufficient to trigger competitive cell elimination (Kucinski et al., 2017). Therefore, it is possible that different mechanisms function in flies and mammals to achieve the same end-result of clearing stressed cells from tissues.

1.5.4. Removal of specific mutations

A final possibility is that cell competition is not triggered in response to general fitness or damage gradients, but is triggered in response to specific types of mutation which are particularly common or deleterious. It is striking that two significant but contrasting groups of mutations induce elimination of cells: those that affect translation, and those that affect polarity. It has been proposed in relation to the former that competition evolved as a mechanism to detected aneuploidy (Baker, 2011), an output of which could be disrupted translational output (discussed further in *Physiological roles of cell competition*). In keeping with this hypothesis, it is possible that specific mechanisms detect specific types of damage. This would provide an explanation for the differences in the behaviour of Myc-overexpressing cells, which have high translation compared with surrounding cells, but not in fast growing cyclin D-overexpressing clones, which would not have elevated levels of translation (de la Cova et al., 2004).

1.6. Stage 2: What are the mechanisms of cell fitness recognition?

A fundamental and intriguing question emerging from the study of cell competition is: how are less fit cells eliminated specifically when in the presence of more fit cells? It is clear from the range of data emerging that different fitness-dependent fate-determining mechanisms occur in

different contexts. This section will be divided into 'passive' competition and 'active' competition. During 'passive' competition, less fit cells undergo death in a heterogeneous environment as they undergo stress they would not normally undergo in a homogenous environment, and no fitness sensing between cells is required. During 'active' competition, cells sense relative fitness levels, and either the more fit cells elicit the elimination of the less fit cells, or the less fit cells initiate their own elimination following detection of surrounding fitter cells. The main difference between the two types of competition is the requirement for a fitness-sensing step, and both mechanisms have been shown to occur during cell competition interactions. Of note, it is not possible to rule out the presence of a recognition step within described models of passive competition, although such a step is not necessary to explain the elimination of less fit cells in these studies.

1.6.1. Passive competition

Competition for BMPs

Early research hypothesised that in the *Drosophila* imaginal wing discs, cells compete for limited levels of the secreted protein Decapentaplegic (Dpp). This protein is a member of the transforming growth factor β (TGFβ) family of growth factors and homolog of the vertebrate bone morphogenetic protein (BMP). The Dpp-competition idea was supported by observations that loser cells of *Minute*- or *Myc*-induced competition express high levels of *Brinker*, a gene negatively regulated by Dpp, prior to their elimination (Moreno and Basler, 2004; Moreno et al., 2002). Deletion of *Brinker* in these losers rescues their elimination (Moreno and Basler, 2004; Moreno et al., 2002). Furthermore, in areas of low Dpp signalling, $Minute^{+/-}$ cells show increased persistence, indicating that areas of widespread Dpp-shortage favour defective cell survival. Deletion of the *Dpp*/BMP receptors is also sufficient to turn cells into losers in both *Drosophila* and in mammalian models of competition (Burke and Basler, 1996; Sancho et al., 2013). However, evidence for Dpp-dependent competition is contradictory. Other groups have reported that Myc-induced competition is not correlated with, nor dependent on, changes in Dpp activity (de la Cova et al., 2004). As the BMP pathway has an important role in cell proliferation, it has been proposed that the rescue of loser cell elimination in response to enhanced Dpp activity compensates for, rather than rescues, competition, since no obvious rescue of cell death occurs (Tyler et al., 2007). Furthermore, in mouse ESCs, ectopic addition of BMPs failed to rescue out-competition of $Bmpr1a^{-/-}$ cells (Sancho et al., 2013). Therefore, while disruption of BMP signalling may affect cell fitness through alteration of proliferation

or metabolism, competition for Dpp/ BMPs does not appear to be the sole mechanism orchestrating cell competition.

Mechanical competition

Cells in an epithelium are subject to a range of mechanical forces which can affect a huge number of cell processes including differentiation, orientation, proliferation, intracellular signalling, and movement (Mammoto and Ingber, 2010). Mathematical modelling has revealed that mechanical forces can contribute to the selection of faster-growing cells in a heterogeneous population (Shraiman, 2005). Specifically, faster growing cells were predicted to compress adjacent slower growing cells and inflict stress sufficient to activate apoptosis. These mathematical predictions have now been validated across various biological systems. Cell crowding induces live cell delamination in the human colon, the zebrafish epidermis, MDCK cell sheets and the *Drosophila* pupal notum (Eisenhoffer et al., 2012; Marinari et al., 2012), and in the pupal notum, slower growing cells are preferentially extruded over fast growing ones (Levayer et al., 2016). Similarly, polarity-deficient MDCK cells are eliminated in culture with their wild-type counterparts through mechanical triggers (Wagstaff et al., 2016). In this system, null mutation of *Scribble* results in moderate p53 activation which renders cells hypersensitive to compaction. As a result, when surrounded by faster growing wild-type cells with higher thresholds for compaction-induced apoptosis, *Scribble*$^{-/-}$ cells preferentially die and wild-type cells colonise the culture (Wagstaff et al., 2016).

Competition for other survival factors

Competition for interleukin-7, IL-7, is thought to mediate selection of young T-cells over old T-cells in the thymus (Martins et al., 2014). IL-7 activates expression of pro-survival protein Bcl-2, and as this cytokine is in limited supply in the thymus, cells with insufficient exposure downregulate Bcl-2 and undergo apoptosis. So far it is unclear what mediates the reduced intake or exposure to IL-7 in older cells (Martins et al., 2014).

1.6.2. "Active" competition

What does a less-fit or more-fit cell look like? And how is this recognised by surrounding cells? These questions lie at the heart of understanding 'active' competition, and so far a few studies have suggested molecular codes which mark fitness status in competition models. Despite this, a code which is conserved across competition models and evolution has remained elusive.

Currently it therefore seems unlikely that cell fitness status converges on a single molecular code which can be read between cells. Rather, evidence collected so far indicates that different mechanisms of cell fitness recognition are used to detect different types of defective cell.

Flower code

The *Flower* genes were proposed to form a fitness fingerprint to convey loser status to neighbouring cells (Merino et al., 2013; Rhiner et al., 2010). *Flower* encodes a transmembrane calcium channel and has three splice isoforms. In competitive environments in *Drosophila*, loser cells upregulate two 'lose' Flower splice isoforms, while surrounding cells express only the 'ubiquitous' isoform. As expression of the Lose isoforms is both sufficient and necessary for loser cell death during competition, the "Flower code" has been proposed to be a cell-surface marker of relative fitness status which allows for the detection and elimination of less fit cells.

Sas-PTP10D cellular crosstalk

Recently, another study revealed a different set of proteins involved in cell fitness recognition in *Drosophila* (Yamamoto et al., 2017). A genetic screen identified that recognition of cell surface receptor PTP10D in *Scribble*$^{-/-}$ cells by wild type cells via the ligand Sas mediates the elimination of these polarity mutants. At the interface of wild-type: *Scribble*$^{-/-}$ clones, Sas and PTP10D relocalise to the lateral surface of the cells and the resultant activation of PTP10D then inhibits EGFR-Ras in the mutant clones which results in the JNK-mediated cell death. In the absence of PTP10D, activation of EGFR-Ras functions alongside JNK to activate F-actin accumulation. This leads to inhibition of the Hippo pathway and subsequent over-proliferation of the polarity defective cells. Therefore, molecular 'recognition' of membrane protein PTP10D on loser cells by Sas on adjacent wild-type cells is required for this form of competition.

Innate immunity pathways

It has been observed that the elimination of loser cells in *Drosophila* requires activation of the Toll-related receptors (Meyer et al., 2014). This has led to the hypothesis that the innate immunity pathway, which is involved in host defence from foreign or altered-self cells, also functions to eliminate mutant cells during cell competition. The secreted cytokine Spatzle is important for the activation of the receptors and subsequent elimination of the cells, indicating

that competition could occur through release of this ligand by winner cells and specific detection by loser cells, or through increased expression of Toll-related receptors by loser cells (Meyer et al., 2014).

In these studies detailing different mechanisms of fitness recognition, it is unclear what drives the expression of cell surface receptors which are specific to low cell fitness. Interestingly, the Sas-PTP10D mechanism driving the recognition and elimination of *Scribble*$^{-/-}$ cells is not shared across all polarity-defective mutant clones. Although other neoplastic polarity mutants show expression of PTP10D in a heterogeneous environment, non-neoplastic polarity mutants such as *stardust*$^{-/-}$ or *crumbs*$^{-/-}$ do not (Yamamoto et al., 2017). This finding therefore suggests that cells integrate information from both the type of mutation and also the potential risk the mutation poses to the tissue when expressing cell-surface signals involved in fitness-recognition. Further understanding of the events responsible for linking reduced cell fitness to expression of 'less fit' markers will provide much needed clarity for this area of cell competition.

1.6.3. Secreted factors or cell-cell contact?

The above describe a wide variety of mechanisms which have so far been described to mediate "classical" cell competition. Naturally, some require cell contact or close range for elimination of loser cells (e.g. cell-cell fitness comparisons and mechanical sensing), whereas others do not (e.g. competition for nutrients/ growth factors). Consistent with this, there have been differing reports concerning the requirement for cell-cell contact during competition. In *Drosophila Minute* models, elimination of cells is greatest in the centre of mutant clones, indicating that here, cell-cell contact is not a requirement of competition (Simpson, 1979). Furthermore, wild-type cells can eliminate non-adjacent *Minute* clones (de la Cova et al., 2004). Such data could point toward passive competition for a shared survival factor, or active competition mediated by secreted factors. In agreement with this, cell culture assays in *Drosophila* and mouse ES models have revealed through conditioned media and transwell experiments that exposure to shared media is sufficient to induce competition (Sancho et al., 2013; Senoo-Matsuda and Johnston, 2007). However, other studies reveal contradictory findings: cell contact has been reported as a requirement of competition between ES cells (Claveria et al., 2013), MDCK cells (Wagstaff et al., 2016), mouse heart cardiomyocyte progenitors (Villa del Campo et al., 2014) and cells in *Drosophila* imaginal discs (Li and Baker, 2007) and the pupal notum (Levayer et al., 2016). One reason for these discrepancies could be the differing mechanisms of cell

elimination in response to different mutations. For example, secreted factor *Notum* is required for the elimination of wild-type cells by over-activation of Wingless signalling in the *Drosophila* imaginal wing disc, but not the elimination of *Minute*$^{+/-}$ cells in the same tissue (Vincent et al., 2011). Determining whether competition is contact-dependent in any given system is likely to provide valuable clues as to the mechanism of fitness sensing.

1.7. Stage 3: What are the processes of cell elimination?

1.7.1. Apoptosis

In many competition models, the elimination of loser cells occurs through activation of apoptosis in the less fit cell type. However, different molecular effectors of apoptosis have been described.

JNK

The JNK pathway responds to a variety of cellular stresses and plays an important role in the activation of apotosis (Dhanasekaran and Reddy, 2008). In various *Drosophila* cell competition models, levels of phospho-JNK are increased in loser cells, and their apoptotic elimination can be blocked by suppression of the JNK pathway (Kolahgar et al., 2015; Moreno and Basler, 2004; Moreno et al., 2002; Suijkerbuijk et al., 2016; Tamori and Deng, 2013). As well as inducing cell death, various other functions have been attributed to JNK activation. For example, JNK activation regulates Hippo which acts to inhibit the proliferation of *Scribble* null cells prior to their elimination (Chen et al., 2012). JNK-dependent non-autonomous signalling in dying cells can also promote the compensatory proliferation of surrounding cells through secretion of paracrine growth-enhancing factors Dpp and Wingless (Ryoo et al., 2004). Similarly, loser cells in the *Drosophila* intestine release cytokine Unpaired-3 in a JNK-dependent fashion (Kolahgar et al., 2015). Unpaired-3 signals to surrounding cells and induces their expansion through the JAK/STAT pathway (Kolahgar et al., 2015). Finally, activation of JNK in the tissue surrounding *Scribble* cells promotes cell engulfment through activation of the *Drosophila* PDGF/VEGF receptor (Ohsawa et al., 2011).

Despite the clear involvement and function of JNK in some competition models, in MDCK *Scribble*$^{-/-}$ cells, competition is JNK-independent (Norman et al., 2012). Furthermore, the involvement of JNK in *Minute*- and *Myc*-induced cell elimination in *Drosophila* is contradictory and not observed in some studies (de la Cova et al., 2004; Tyler et al., 2007).

One suggested reason for this discrepancy could be the method of clone generation. Groups which report a role for JNK in elimination of less fit cells use heat shock to generate clones of mutant cells in flies (Moreno et al., 2002; Moreno and Basler, 2004; Tamori and Deng, 2013; Kolahgar et al., 2015; Suijkerbuijk et al., 2016). Since JNK responds to a variety of stresses, it is possible that heat shock activates JNK signalling (Gibson and Perrimon, 2005).

Azot

Azot encodes a calcium-binding cytoplasmic protein and is the first protein to be described as having an exclusive role in the elimination of cells during cell competition (Merino et al., 2015). While various competitive scenarios in *Drosophila* result in the upregulation of Azot protein levels in loser cells, deletion of *Azot* prevents the elimination of loser cells. Furthermore, its overexpression results in increased elimination of loser clones. Azot has therefore been proposed to act as a cell-fitness checkpoint which is involved in deciding cell fate in a competitive scenario (Merino et al., 2015). It is so far unclear if *Azot* function is conserved in mammals.

NF-kB

NF-kB family members have a role in the elimination of loser cells downstream of the Toll-related receptors in *Drosophila* (Meyer et al., 2014). In *Myc*- and *Minute*- induced competition, loser cells show increased activation of NF-kB homologs specifically in a competitive scenario. This is dependent on expression of the Toll-related receptor. Interestingly, each competitive scenario relies on different NFkB family members during cell death: elimination of *Minute* losers occurs through Relish activation, whereas elimination of low-Myc cells occurs through Dorsal and Dorsal-related immunity factor activation (Meyer et al., 2014).

1.7.2. Cell engulfment

It has been proposed that loser cells in *Drosophila* imaginal wing discs are eliminated through engulfment by surrounding winner cells (Li and Baker, 2007). Support for this theory comes from the observation that corpses of eliminated loser cells can be seen in adjacent wild-type cells, and that competition is inhibited by knockdown of cytoskeletal regulators which are required for engulfment. Similarly, out-competition of wild-type cells by cells overexpressing EGFR and the microRNA miR-8 relies on engulfment proteins (Eichenlaub et al., 2016). However, since the cytoskeleton has many cellular functions, the rescue of competition

following alteration of cytoskeletal components may be due to engulfment-independent cytoskeletal roles, such as an involvement in sensing mechanical stress (Wagstaff et al., 2016). Furthermore, other reports have contested a role for engulfment proteins in competition in developing *Drosophila* tissues (Lolo et al., 2012). In this study, clearance of apoptotic debris is attributed to hemocyte activity rather than engulfment by wild-type cells.

Cell engulfment has also been implicated in cell competition between breast or pancreatic cancer cell lines and non-tumourigenic cell lines derived from the equivalent tissue. This process, named 'entosis', is dependent on the cytoskeleton and correlates with mechanical deformability (Sun et al., 2014b). Specifically, cells which are less deformable are engulfed by more deformable cells.

1.7.3. Cell senescence

Senescence is a form of cell cycle arrest that occurs in response to a variety of stresses including telomere attrition, ROS damage and oncogene-activation (Munoz-Espin and Serrano, 2014). Outcompeted hematopoietic stem cells are eliminated from the functioning pool of cells through activation of senescence-like programs (Bondar and Medzhitov, 2010). The authors here suggest that *in vivo*, the senescence-like phenotype permits continued proliferation and function of cells but marks them as damaged and so acts to facilitate gradual replacement by fitter cells over time. It will be interesting to investigate in future years to what degree physiological senescence occurs as an outcome of cell competition.

1.8. Evidence for endogenous competition

1.8.1. During embryogenesis

So far, most cell competition research has been using experimentally-induced competition models which have given insight into the occurrence and mechanisms of competition, but have failed to reveal the endogenous rate or function of the process. Part of the difficulty is that there is no specific marker for competition. For example, analysis of heterogeneity of Myc levels in early mouse development implies that endogenous cell competition occurs at a high rate in these tissues (Claveria et al., 2013). However, as Myc has other functions, it is unclear whether low Myc cells represent those which are less fit and are being subjected to outcompetition, or whether the fluctuation of Myc levels is stochastic or related to another cell function. In fact, the authors report that preventing cell competition by ectopic expression of apoptosis inhibitor

p35 yields healthy mice with no obvious phenotypes, suggesting that the persistence of "less-fit" low-Myc cells has no negative impact on organism health. Clearly, a competition-specific marker is required to fully understand rates and roles of cell competition, and recent work has started to address this (Merino et al., 2015).

Perhaps more convincing evidence supporting endogenous competition in embryo development comes from studies of human embryos used for *in vitro* fertilisation (IVF). Analysis of chromosomal mosaicism indicates that 50-80% of preimplantation embryos have some degree of mosaic aneuploidy (van Echten-Arends et al., 2011). Strikingly, after known mosaics are transferred to mothers, healthy babies are born which lack aneuploid cells, indicating that the embryo is able to correct itself from errors (Greco et al., 2015), potentially through cell competition. This correction has also been demonstrated in mouse embryos (Sancho et al., 2013; Bolton et al., 2016). Although further research is required prove that cell non-autonomous interactions are at play in human embryos rather than autonomous cell death, taken together, these studies point toward a role for endogenous cell competition in safeguarding the developing foetus from aneuploidy.

1.8.2. Throughout life

Few studies have been carried out into endogenous competition rates throughout life. However, monoclonality occurs in the hematopoietic niche with age, and given the demonstrated susceptibility of HSCs to competition (Bondar and Medzhitov, 2010), this could be a result of the emergence of highly competitive cheater mutations, or through the selective advantage gained by certain clones (Busch et al., 2015; Sun et al., 2014a).

1.9. Evolution and the physiological role of "classical" cell competition

A final aspect of cell competition which is part of ongoing investigation is understanding the precise physiological roles of the process. Furthermore, while 'passive' cell competition could be seen as a facet of multicellularity, there is interest in understanding why 'active' forms of cell competition evolved. Several theories have been put forward to suggest potential roles of cell competition and these will be discussed in this section.

1.9.1. Cell cooperation or cell competition?

It has been proposed that cell competition has roots at the dawn of eukaryotic evolution, and acts to safeguard multicellularity and cell cooperation (Amoyel and Bach, 2014; Dejosez et al., 2013). With multicellularity comes a change in the way cells function: cells which existed alone were now required to associate and cooperate with each other to form a single larger cohesive unit. Cells which fail to cooperate pose a threat to the fitness and survival of the organism, cancer being an obvious example of loss of this cooperative regulation. Therefore, cell competition could have evolved as a mechanism to prevent propagation of cells which pose a threat to cell cooperation, such as those which are functioning sub-optimally or are mispatterned. In this 'cell cooperation' line of research, cooperation 'cheaters' have been described in chimeric multicellular amoeba and mouse embryos (Dejosez et al., 2013; Santorelli et al., 2008). These mutant cells contribute in disproportionately high levels to the germline and are reminiscent of supercompetitors. Therefore, it is possible that cell competition mechanisms could actually stem from mechanisms which ensure cell cooperation in multicellular life. Indeed, various genes identified in an embryogenesis cheater screen are previously described regulators of cell competition, including p53, cMyc, and JNK target c-Jun (Dejosez et al., 2013), indicating that there may at least be significant overlap between the two processes.

1.9.2. Organ size regulation

In *Drosophila*, overexpression of Myc in all cells of a tissue results in an increase in the size of adult flies (de la Cova et al., 2004). However, heterogeneous overexpression of Myc results in apoptosis of low-Myc cells, and as a result the large-size phenotype is rescued and flies are indistinguishable from normal flies. This observation led to the proposal that cell competition is a mechanism that evolved to maintain and regulate organ size, and acts to counter the increase in growth of one highly proliferating population by the death of another. This hypothesis is attractive as many models of cell competition are phenotypically silent. However, as previously mentioned, not all faster growing populations of cells induce competition, and in these cases organ size is increased (de la Cova et al., 2004). Therefore the benefit of cell competition for regulating organ size in response to a small number of mutations is not clear. Additionally, this theory does not explain why slower growing defective cells are eliminated from tissues, as their persistence should not adversely affect tissue size. Finally, inhibition of death in the developing wing disc does not alter compartment size (Martín et al., 2009). Clearly,

this hypothesis explains some features of cell competition but alternative or additional theories are required to explain all features.

1.9.3. Optimisation of tissue fitness

Cell competition has been proposed to act as a quality control mechanism which selects against damaged or suboptimal cells both during development and throughout organism lifespan (Morata and Ripoll, 1975). In this way, cell competition could safeguard tissue and organismal fitness. This is supported by the fact that a large range of less-fit cell types have been shown to be eliminated in a context-dependent fashion in flies and mammals.

Compelling functional evidence supporting this hypothesis has been performed in flies, in which cell competition rates can be tweaked by altering the levels or outcome of *Azot* activation (Merino et al., 2015). Deletion of *Azot*, and thus inhibition of cell competition, results in reduced lifespan and increased signs of tissue degeneration in fly wings and brains. Furthermore, tissue damage following UV exposure is exacerbated. The results therefore indicate that cell competition involving Azot clears damaged cells, enables buffering of tissues following damage, and provides a protective effect against neurodegeneration and other age-related ailments. Remarkably, increasing cell competition through expression of another copy of Azot, or replacement of the gene with apoptotic gene Hid, results in an increase in both lifespan and healthspan, as shown by a decrease in wing aberrations and neurodegenerative vacuoles. Although similar findings are yet to be confirmed in mammalian systems, this study provides support for a role for cell competition in the clearance of damaged cells which increases healthspan.

1.9.4. Guarding against aneuploidy

Aneuploidy is a common characteristic of cancers and is thought to be a driver of tumourigenesis by promoting cellular heterogeneity and aiding tumour immune system evasion (Davoli et al., 2017; Sansregret and Swanton, 2017). Cell competition has been proposed to act as a method to detect and eliminate aneuploid cells (Baker et al., 2011). This hypothesis sprung from the fact that ribosomal genes are spread out across different chromosomes (Marygold et al., 2007). As a result, cells with unequal numbers of chromosomes would produce unbalanced levels of ribosomal subunits, leading to their stoichiometric imbalance and defective translation. The fact that several of the initial cell competition-triggering mutations described in *Drosophila* converge on translation led to the proposal that translation efficiency could be a

readout of aneuploidy, and by eliminating cells with reduced translation capability, aneuploid cells would be removed. Further support for this came from the finding that tetraploid cells are eliminated during early mouse development by cell competition (Sancho et al., 2013). However, tetraploid cells should in theory have balanced ribosomal stoichiometry, and as a result the competition 'readout' for aneuploidy is not clear. The mechanisms leading to cell competition evasion by aneuploid tumours are also unknown.

1.9.5. As a tumour suppressive mechanism

There is expanding evidence from both *in vitro* and *in vivo* studies to suggest that cell competition has a tumour suppressive function. First, the process prevents the propagation of polarity mutant cells. These genes are tumour suppressors in *Drosophila* and humans, and mutant cells are eliminated by wild-type cells in mammalian culture (Norman et al., 2012) and in *Drosophila* tissues (Agrawal et al., 1995; Brumby and Richardson, 2003; Menendez et al., 2010; Yamamoto et al., 2017). If the elimination of polarity mutant cells is prevented by JNK inhibition, tumours develop (Brumby and Richardson, 2003; Igaki et al., 2009), indicating that cell competition has a potent tumour suppressive role. Interestingly, expression of active oncogenes Ras^{V12} or $Notch^{ACT}$ in these cells increases cell proliferation through repression of Hippo and allows some clones to escape death by cell competition and grow into tumours (Brumby and Richardson, 2003; Menendez et al., 2010). These results suggest that cell competition can prevent the propagation of stray polarity mutant cells, but that its efficacy, at least in *Drosophila,* declines beyond a threshold of clone size, perhaps due to the generation of a cell competition-insensitive tumour microenvironment. However, as some Ras^{V12}-transformed polarity mutant cells are still sensitive to competition, it could be productive to identify whether cell competition mechanisms can be 'boosted' in surrounding wild-type tissue in order to treat certain types of cancers.

While these results are interesting and support a case for a tumour-suppressive role of cell competition, it is puzzling to imagine why they could have evolved during fly development, since spontaneous mutagenesis and tumours only occur in aged fly tissues (Siudeja et al., 2015). Without this selection pressure, it is unlikely that cell competition evolved as a potent mechanism to prevent the propagation of oncogenic cells in flies. Tumour-suppression could therefore be a fortunate outcome rather than a driver of cell competition evolution.

In vivo evidence in mice suggests cell competition occurs in the thymus to protect from tumours resembling human T-cell acute lymphoblastic leukaemia (Martins et al., 2014). In this instance, a fresh supply of progenitor T-cells continuously enter the thymus from the bone marrow and outcompete 'older' thymus-resident competitors. Blockage of this replacement by bone marrow progenitors, and therefore of cell competition, leads to tumour development. Although it is unclear why older T-cells have such high disposition for leukaemia development, this study reveals that cell competition has an important physiological role in maintaining the renewal of the cells in the thymus which prevents the development of tumours.

1.9.6. As a tumour-promotion mechanism

Conversely, cell competition could be a potent tumour promotion mechanism when exploited by cancer cells. Initial evidence for this comes from the numerous oncogenes and tumour suppressors which have been implicated in supercompetition, but now a body of evidence is growing for a role for cell competition in promoting tumour development at the early stages of cancer cell expansion, during later stage tumour growth, and in metastasis.

At the earliest stages of tumourigenesis, cell competition has been proposed to have a role in "field cancerization", a phenomenon described in clinical settings where expansion of a clone of pre-neoplastic cells predisposes an area to tumourigenesis (Braakhuis et al., 2003; Slaughter et al., 1953). Cell competition has been described in the intestinal crypt, where cells with oncogenic mutations expand at the expense of normal cells (Snippert et al., 2013; Vermeulen et al., 2013), and in the oesophagus, where Notch-mutant cells with high levels of self-renewal induce differentiation in surrounding wild-type cells through stem cell competition (Alcolea et al., 2014). In these cases, cell competition contributes to the generation of an expanded area of pre-neoplastic cells from which tumours are likely to arise.

At later stages of cancer development, cell competition can lead to promotion of tumour growth. This has been shown in *Drosophila* through analysis of clones of cells with depleted *Notch*, a gene required for cell differentiation, or *APC1* and *2*, genes widely implicated in sporadic colorectal cancer occurrence in humans (Fodde, 2002; Suijkerbuijk et al., 2016). Intestinal stem cells with depleted Notch compress neighbours and trigger cell death. Similarly, deletion of *APC1/2* in clones of cells results in the formation of adenomas which grow at the expense of surrounding wild-type tissue. Notably, preventing death of surrounding cells contains the growth of the cancerous cells, indicating that cell competition provides a

mechanism for tumour expansion. Interestingly, in both cases the death of surrounding cells results in cell non-autonomous signalling which contributes to tumour growth, demonstrating that cell competition in this instance not only results in the clearance of space for tumour expansion but also aids proliferation of the winner population through paracrine signalling from the surrounding damaged tissue.

Another study has revealed that cell competition is important in both the development and metastasis of neoplastic tumours (Eichenlaub et al., 2016). Overexpression of EGFR with miR-8 in clones of cells in the *Drosophila* imaginal wing disc results in the formation of highly aggressive metastatic tumours which contain "giant" polyploid cells. These giant cells are formed through engulfment of surrounding wild-type cells, and inhibition of engulfment prevents both giant cell formation, neoplastic transformation, and metastasis (Eichenlaub et al., 2016). These studies indicate that cell competition plays a crucial role in the expansion of some tumours, and that inhibition of competition in this scenario could be a promising therapeutic intervention.

There is also evidence for a role of cell competition in promoting tumour development in humans through use of *in vitro* cell competition models. Human cancer cells compete with non-cancer cells through a type of cell engulfment called entosis (Sun et al., 2014). A range of cancer lines outcompete non-tumourigenic lines through entosis, and overexpression of active oncogene KrasV12 is sufficient to induce competitive interactions between previously non-tumourigenic lines. Competitive behaviour in this instance is triggered by differences in mechanical deformability, a property which Kras can alter (Sun et al., 2014). This study reveals that in human cancers, competitive behaviour could promote tumour growth and metastasis.

1.10. Other subgroups of cell competition

1.10.1. Neurotrophic theory

Another branch of cell competition is the neurotrophic theory which has stemmed from the study of neuronal death during development (Oppenheim, 1985; Raff, 1992). At a crucial time in nervous system development, when target cells are innervated, a wave of cell death, or 'neuronal culling', occurs. In this process, nearly half of the initially produced neurons die. The neurotrophic theory posits that this death is mediated by competition for limited amounts of nerve growth factor (NGF) from target cells which supports the survival of only a limited

number of neurons (Oppenheim, 1981; Raff, 1992). The harsh competition is thought to not only ensure that the correct number of neurons innervate each target cell, but also that the ones that do are the fittest. The outcome of these trophic interactions is aided by release of a 'death signal' from cells receiving high levels of NGF to promote death of cells receiving low levels of NGF (Deppmann et al., 2008). Neuronal competition differs from "classical" competition as it is not obviously dependent on fitness differences between cells. It is still unclear exactly what determines the competitive advantage of the surviving neurons, but it is thought to be cells with more growth factor receptors, or more efficient transduction of signals.

1.10.2. Epithelial defence against cancer

In the 1960s, analysis virus-transformed, oncogenic, or carcinogen-treated hamster and rat fibroblasts revealed context-dependent cell behaviour. Alone, these cells proliferate and form multi-layered colonies; however, when cultured with non-transformed cells, their proliferation is impeded (Borek and Sachs, 1966; Stoker, 1964; Stoker et al., 1966). Although these studies describe non-autonomous changes in cell behaviour, the mechanisms and triggers of the process were not studied in great detail.

Much more recently, similar phenomena has been revisited by exploring cell-non-autonomous interactions in cultured MDCK cells and *in vivo* in zebrafish and mice (Hogan et al., 2009; Kajita et al., 2010; Kon et al., 2017). Cells carrying oncogenic mutations RasV12 and v-Src are eliminated specifically when surrounded by wild-type cells (Hogan et al., 2009; Kajita et al., 2010; Kon et al., 2017). Since surrounding wild-type epithelial cells play an active role in this process through alteration of cytoskeletal components, this process has been named 'Epithelial Defence Against Cancer' (Kajita et al., 2014). Interestingly, the elimination of loser cells does not occur through apoptosis but occurs through extrusion from the epithelium. Cell adhesion is important for this event as knockdown of adherens junction protein E-cadherin inhibits cell extrusion. Although in most cases delaminated cells will die from lack of cell contact, survival could occur if cells have acquired mutations which override this (Kajita et al., 2010).

Studies of organotypic culture of human breast epithelial cells have revealed that apical extrusion of oncogene ErbB2-overexpressing cells also occurs in human cells (Leung and Brugge, 2012). Although this extrusion can be beneficial and result in death of the oncogenic cell through anoikis, if the cell has acquired mutations which allow it to survive in the absence

of cell-cell interactions, the extrusion could promote cancer cell outgrowth and metastasis by removing mechanical or signalling epithelial limitations (Leung and Brugge, 2012).

1.10.3. Stem cell neutral drift

During neutral stem cell drift between adult stem cells, all cells are equally likely to be lost to differentiation or to self-renew, and it is thought that random fluctuations in signalling factors mediate the process (reviewed in Stine and Matunis, 2013). However, over time stem cell niches become monoclonal. Interestingly, cells at the border of intestine stem cell niches have an increased probability of differentiating compared to stem cells in the centre (Ritsma et al., 2014). It will be interesting to identify in future years whether 'neutral' cell competition really is stochastic, or whether through fitness comparison mechanisms, more fit cells preferentially self-renew to maintain the stem cell niches with maximal fitness and minimal damage. It is feasible that stem cells at the borders of niches would incur elevated levels of damage which could provide an explanation for their increased propensity to differentiate.

Since stem cell competition is thought to be mediated by exposure of cells to either survival or differentiation factors, another role for the process in this setting could be to allow fine-tuned regulation of stem cell behaviour in a way that is coupled to organismal need. For example, in the *Drosophila* midgut, stem cell regeneration is tightly coupled to nutrient availability (O'Brien et al., 2011). After periods of feeding, elevated insulin levels promote symmetric renewal and heightened proliferation which drives organ growth. Competition for survival factors, even in fitness-independent 'neutral' instances, would therefore permit fine-tuned adaptive responses which enhance organismal robustness in the face of environmental volatility. Finally, stem cell competition could also ensure the maintenance of appropriate cell ratios when multiple cells occupy the same niche, such as that orchestrated by JAK/STAT signalling in the *Drosophila* testes (Issigonis et al., 2009).

1.10.4. Cancer-host competition

The study of cell non-autonomous interactions in cancer has revealed cell competition-like mechanisms which are mediated through nutrient availability. In glioblastomas and sarcomas, cancer cells take up large amounts of glucose and effectively starve surrounding tissue and immune cells (Chang et al., 2015; Flavahan et al., 2013). This passive mechanism of competition exploited by cancer cells promotes their expansion and immune evasion.

1.11. Aims of study

Cell competition has been shown to act during early mouse embryogenesis (Claveria et al., 2013; Sancho et al., 2013). However, neither the molecular mechanisms involved in cell competition nor its precise physiological roles at this stage are fully understood.

The aims of this study were as follows:

1. To identify whether the induction of senescence occurs in loser cells during ESC competition, and whether senescence has a role in promoting the elimination of loser cells.
2. To mine microarray gene expression data gathered from competing ESCs to assess other possible molecular mechanisms involved in cell competition.
3. To use information gained from further understanding of the molecular mechanisms of cell competition to assess the physiological roles of the process during mouse embryogenesis.

We use two different model systems to examine cell competition *in vitro* and *in vivo*.

1.11.1. *In vitro* system

Culture conditions

Our *in vitro* cell system is designed to mimic the embryo at either E3.5, where cells are maintained in a pluripotent state, or the onset of gastrulation (E6.5), where differentiation is triggered. Pluripotency is maintained by culturing cells in ESC media in the presence of serum and leukaemia inhibitory factor (LIF). In order to simulate differentiation, cells are cultured in serum-free N2B27 media containing numerous growth factors. Since cell competition only occurs after cells exit the pluripotent state, competition assays are performed by seeding cells into N2B27 media.

Loser cell lines

To model cell competition, we use two 'loser' cell lines: bone morphogenetic protein (BMP) signalling-defective cells *Bmpr1a*$^{-/-}$ cells and tetraploid (4n) cells. Both of these cell types are viable when cultured alone or with other less fit cells, but are eliminated through apoptosis when cultured with wild-type cells (Sancho et al., 2013).

BMPs belong to the transforming growth factor (TGF) superfamily and have multiple roles in embryogenesis, particularly in mesoderm formation and cardiac development (Gaussin et al., 2002; Mishina et al., 1995). *Bmpr1a*$^{-/-}$ cells, which have null mutations in the bone morphogenetic protein receptor 1a gene, can transduce BMP signals but have reduced levels of pathway activation compared with wild-type cells (Sancho et al., 2013). These cells have a slight growth defect during differentiation but are viable and can differentiate into all three germ layers (Fig. 1.4; Sancho et al., 2013). However, from around day 3 in co-culture with wild-type cells, they start to be eliminated through apoptosis (Sancho et al., 2013; Fig. 1.4). Notably, use of caspase inhibitors prevents the out-competition of loser cells indicating that the reduction in *Bmpr1a*$^{-/-}$ cell numbers when in co-culture with wild-type cells is due to an induction of apoptosis rather than growth differences (Sancho et al., 2013).

4n cells have double the amount of chromosomes compared to wild-type cells. Like *Bmrp1a*$^{-/-}$ cells, 4n cells proliferate slowly in culture alone but are eliminated through apoptosis when in culture with wild-type cells (Sancho et al., 2013). When studying mechanisms that are involved in mediating competition, we study molecular changes that occur at day 3 since this is the day at which loser cells start to be eliminated (Fig. 1.4).

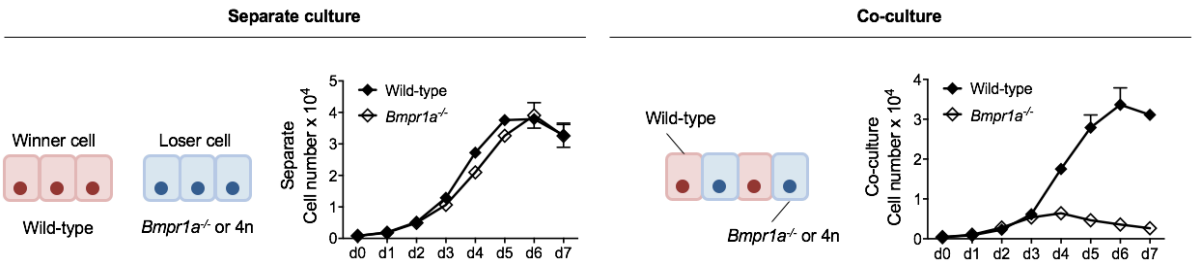


Figure 1.4: In vitro system of cell competition.

To study cell competition *in vitro,* wild-type and defective cells are cultured separately and together and cell ratios and counts are measured over 7 days in N2B27 media. n=2; error bars denote SEM.

1.11.2. *In vivo* system

To study cell competition *in vivo,* we generate chimeras of wild-type/ tetraploid cells. Whole tetraploid embryos can survive until birth (Kaufman, 1991, 1992; Snow, 1975), and gastrulate at a normal stage (Henery et al., 1992). However, when tetraploid cells are present in diploid embryos, they are efficiently eliminated (Bolton et al., 2016; Eakin et al., 2005; Sancho et al., 2013). When assessing molecular changes that occur during competition, we analyse chimeras

at E5.5, prior to the stage that tetraploid cells are eliminated. When assessing cell death that occurs as a result of competition, we analyse chimeras at E6.5.

Chapter 2: Materials and methods

2.1. Cell culture techniques

2.1.1. Cell passaging

Wild-type E14Tg2a.IV embryonic stem cells were a gift from Prof. A. Smith; *Bmrp1a*$^{-/-}$ cells and were a gift from Yuji Mishina (Qi et al., 2004; Sancho et al., 2013). Tetraploid cells were generated through the fusing of two wild-type cells by electrophoresis and were determined to have 80 chromosomes through karyotyping analysis (Sancho et al., 2013).

Cells were cultured in GMEM-BHK1 (Invitrogen) supplemented with 10% FCS, 2mM glutamine (Invitrogen), MEM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM β-mercaptoethanol (Invitrogen) and 100 units/ml leukaemia inhibitory factor (LIF; generated and tested in the lab). ESCs were split every 2-3 days, maintained on gelatin-coated flasks, and media was changed daily.

2.1.2. Competition assay

Cells were seeded onto fibronectin-coated plates at a concentration of 2.05×10^4 cells/ cm^2 either separately or mixed for co-cultures at a 50:50 ratio. Cells were grown in N2B27 media (Neurobasal media; DMEM F12 media; 0.5x B27 supplement; 0.5x N-2 supplement; 2-mercaptoethanol; 0.2mM glutamine; all Invitrogen) to allow for differentiation. At the indicated timepoints, cells were counted using ViCell Counter and Viability Analyser (Beckman Coulter), and proportions of each cell type in co-cultures were determined using LSR II Flow Cytometer (BD Bioscience). Sytox blue (Invitrogen) or propidium iodide (Sigma) dyes were used to stain for dead cells.

2.1.3. FACS sorting

Cells were sorted for protein or mRNA extraction using BD FACS ARIA II (BD Biosciences). Cultured cells were detached from plates using accutase to form a single-cell suspension. Following centrifugation, cell pellets were resuspended in PBS containing 1% BSA and propidium iodide to detect dead cells. Cell sorting was performed at room temperature. FACS gates were applied to remove cellular debris, cell doublets and PI-positive dead cells, and were sorted on the basis of GFP expression. A minimum of 1.5 million cells per sample were

collected into N2B27 media. Sorting was performed by James Elliot and Thomas Adejumo at the CSC FACS Facility.

2.1.4. Karyotyping analysis

Fresh media was applied to actively growing ES cells (at ~50-70% confluency) for 1 hour before adding colcemid, a microtubule inhibitor that arrests cells in metaphase, for 1 hour. Cells were then trypsinised and centrifuged before being resuspended in a dropwise fashion in 5.6% w/v potassium chloride solution. Cells were incubated at room temperature for 10 minutes prior to being centrifuged and resuspended in cold fixative solution (3 parts methanol; 1 part acetic acid) and incubated for 10 minutes. Cells were centrifuged and resuspended in either KCl or fixative 3 more times before being dropped on to acid-washed glass slides from a height of 1 metre. Metaphase spreads were counterstained using 10% Giemsa in PBS and were imaged on a widefield microscope (Leica).

2.1.5. Drug treatments

Rapamycin (Cayman Chemical) was used at a concentration of 1μM for acute (2h) treatments and 5-50nM for longer treatments. Nutlin-3a (BioVision) was used at a concentration of 5-10μM for 8 hours. Z-VAD-FMK caspase inhibitors (R&D Systems) were used at a concentration of 100μM for 24 or 48 hours, as indicated. In all cases the appropriate volume of DMSO was used for control treatment groups.

2.2. Mouse procedures and embryology methods

2.2.1. Mouse maintenance

Embryos were harvested from F1 (C57BL/6×CBA) or C57BL/6×F1 crosses. All mice were maintained on a 10 hr-14 hr light-dark cycle. Noon of the day of finding a vaginal plug was designated E0.5.

2.2.2. Chimera generation

E2.5 embryos were flushed from oviducts in M2 (Sigma) and cultured in BlastAssist (Origio) under embryo-tested mineral oil (Sigma) at 37°C and 7% CO2 in air. ESCs (3-8) were injected via a laser-generated perforation in the zona pellucida using XYClone (Hamilton Thorne Biosciences). All mice were maintained and treated in accordance with the Home Office's Animals (Scientific Procedures) Act 1986. Chimeras were generated through collaboration

with Jennifer Nichols (University of Cambridge) or Sara Pozzi and Juan-Pedro Martinez Barbera (UCL).

2.2.3. Embryo staining

Embryos were dissected in M2 media (Sigma) and fixed using 4% PFA with 0.01% Triton X-100 and 0.1% Tween for 10 minutes. Permeabilisation was performed with 0.5% Triton X-100 in PBS for 20 minutes, and embryos were blocked using 2% horse serum in PBT for 45 minutes. Primary antibodies (Appendix Table 3) were incubated with embryos in blocking solution overnight at 4°C. Following another blocking step, secondary antibodies (Appendix Table 3) were applied for 1 hour at 4°C. Hoescht was used to stain nuclei.

β-galactosidase staining was carried out using β-galactosidase staining kit (Cell Signalling). Embryos were fixed in 2% formaldehyde, 2% glutaraldehyde for 20 minutes (E5.5-8.5), 30 minutes (E9.5) or 45 minutes (E12.5) at room temperature. Embryos were incubated in β-galactosidase staining solution containing 1mg/ml X-gal was buffered to pH 6.0 overnight at 37°C. Following washes, embryos were imaged using a Leica dissection microscope.

2.2.4. Microsurgical manipulation of embryos for RNA

Embryos at E7.5 were dissected from mouse uteri and cut at the embryonic/ extra-embryonic boundary. Each section was pooled in each litter for analysis of embryos and RNA was collected and subjected to qPCR analysis, as described in *qPCR*.

2.2.5. Embryo immunohistochemistry

Embryos were soaked first in 30% sucrose/ PBS at 4°C and then in 7.5% gelatin/ 15% sucrose/ PBS at 37°C, before being transferred to room temperature to allow the gelatin/sucrose mixture to solidify and blocks to form. Blocks were transferred to 2-methylbutane at -70°C and stored at -80°C before sectioning. Sectioning was performed on a cryostat (Leica) using glass blades. Immunohistochemistry procedures were performed by Aida di Gregorio (Imperial College London).

2.2.6 Embryo genotyping

Embryos were digested in 25µl TE-TWEEN lysis buffer (50mM Tris-HCl pH 8-8.5; 1mM EDTA; 0.5% TWEEN-20) with 1µl proteinase K (20mg/ml stock) overnight at 55°C.

Following heat inactivation at 95°C for 5 minutes, 2µl of digest was used for PCR reaction. PCR reaction contained 0.2mM dNTPs (Roche), Amplitaq PCR buffer (Thermo Fisher Scientific), 1µM forward/reverse primer and 1 unit Amplitaq DNA polymerase (Thermo Fisher Scientific) in a final volume of 50µl. Genes were amplified using the following PCR program: 95°C, 5 minutes; 35 amplification cycles of 94°C 30 secs, 60°C 30 secs, 72°C 45 secs. Bands were resolved under UV light following electrophoresis on an ethidium bromide-stained 1% agarose gel.

2.3. Methods to detect gene expression

2.3.1. Microarray

Microarray sample preparation was performed by Margarida Sancho. RNA extraction was performed using Trizol (Invitrogen) obtained by Rneasy RNA isolation kit (Qiagen) and DNA removed by RNAase-free Dnase (Qiagen). Microarray was carried out using mouse gene 1.0 ST Array system (Affymetrix) at UCL Genomics at the Institute of Child Health, UCL. Data normalisation was carried out by Gopuraja Dharmalingam (Imperial College London) and analysed using Ingenuity Pathway Analysis (Qiagen Bioinformatics).

2.3.2. qPCR

Cells were trypsinised, centrifuged at 1400rpm for 4 minutes and RNA from the pellet was extracted using Rneasy RNA isolation kit (Qiagen). DNA was removed from samples by incubation with Rnase-free Dnase (Qiagen). RNA concentration was determined by Nanodrop spectrophotometer (Thermo Fisher Scientific).

cDNA was synthesised from 1.5µg RNA using Superscript III reverse transcriptase (Invitrogen). First, 1.5µg RNA and random nonamers (to a concentration of 5µM in H_2O) were mixed and incubated at 65°C for 5 minutes before slowly cooling to 4°C. Second, a mixture of 200 Units SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), 40 Units Protector Rnase Inhibitor (Roche), 1× Reverse Transcriptase buffer (Thermo Fisher Scientific), 20mM DTT, 20mM dNTPs was added to each RNA reaction. cDNA was synthesised using the following program: 5 min, 25°C; 90 min, 50°C; 15 min, 70°C and diluted 1:10 in H_2O prior to qPCR amplification. Negative control samples contained identical reaction mixtures without the reverse transcriptase enzyme.

Assessment of mRNA expression by qPCR was performed in 384-well plates (Thermo Fisher Scientific) using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Reaction mixes containing 1μl diluted cDNA (as above), 1× SYBR Green Master Mix (Qiagen), 330nM forward/ reverse primer (Sigma; Appendix Table 4) in H_2O were added to wells. Reactions were performed in triplicate and one reaction of negative control cDNA was used for each sample and primer. PCR program used was as follows: 95°C, 15 min; 40 cycles of 94°C 15 sec, 60°C 30 sec, 72°C 30 sec, plate read. Measurements were normalised to housekeeping gene β-actin, as this gene was found to yield most consistent Ct values when compared to other housekeeping genes.

2.4. Methods to detect protein levels

2.4.1. Western blotting

Cells lysates were collected using Laemmli buffer (0.05M Tris-HCl at pH 6.8, 1% SDS, 10% glycerol, 0.1% β-mercaptoethanol) and quantified using BCA quantification (Thermo Fisher). 30μg of protein was loaded into wells of Criterion XT pre-cast gels (BioRad) and ran for 80 minutes at 150V in XT MOPS buffer (BioRad). Proteins were transferred to a PVDF membrane (Thermo Fisher Scientific) by wet transfer for 1 hour at 100V. After blocking in 5% milk in TBS-0.1% Tween (TBS-T) for 1 hour, membranes were incubated with antibodies (Appendix Table 3) in TBS-T, 5% milk overnight. The following day, membranes were incubated with secondary HRP-conjugated antibodies (Appendix Table 3) in TBS-T, 5% milk. Chemiluminescence was assayed using ECL detection reagents (Promega) and membranes were exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

2.4.2. Immunocytochemistry

Cells were grown on fibronectin-coated glass coverslips and fixed in 4% PFA for 10 minutes, permeabilised with 0.4% Triton X-100 for 5 minutes, and blocked with 10% BSA (Sigma), 0.1% Triton X-100 for 1 hour. Staining with primary antibodies (Appendix Table 3) was performed overnight at 4°C in 1% BSA, 0.1% Triton X-100. After 3 washes in PBS, secondary antibodies (Appendix Table 3) and Hoescht (Invitrogen) were applied for 1 hour. Coverslips were then mounted onto glass slides using Vectashield (Vector Laboratories). All images were captured on a Zeiss LSM-780 confocal microscope and quantification was performed using ImageJ. Levels of cytoplasmic pS6 were quantified by measuring average fluorescence in the

two pixels surrounding cell nuclei, using DAPI as a mask. Levels of nuclear p53 were quantified by measuring average fluorescence in cell nuclei, using DAPI as a mask.

2.4.3. Flow cytometry staining

All flow cytometric analysis was performed using a BD LSRII flow cytometer and analysed using FlowJo software (both BD Biosciences).

Intracellular staining by flow cytometry

Cells were detached from plates using accutase and fixed in 7.4% formaldehyde in N2B27 media for 10 minutes. Permeabilisation was carried out by dropwise addition of ice-cold methanol and incubation on ice for 20 minutes. Cells were blocked for 10 minutes using 1% BSA/PBS. The primary antibody (Appendix Table 3) was incubated with cells in 1% BSA/ PBS for 1 hour at room temperature. Following three rounds of washing, the secondary antibody was applied (Appendix Table 3) for 30 minutes. Cells were passed through a flow cytometry following two rounds of washing. Gates were applied to remove cell debris and cell doublets.

GFP analysis of live cells

To assess competition GFP ratios, cells were detached from plates using trypsin, quenched with media and equal volumes of cells cultured separately (eg. Wild-type + *Bmpr1a*$^{-/-}$ cells) were mixed together into one tube. Following centrifugation and resuspension, cells were stained with propidium iodide to detect dead cells. Ratios of cells in separate and co-culture were determined. Gates were applied to remove cell debris, doublets, and PI-positive cells.

CD31 staining

CD31 staining was performed in live cells. Cells were detached from plates using accutase. Following resuspension and centrifugation, the antibody (Appendix Table 3) was incubated with cells for 45 minutes at 4°C. Cells were washed prior to re-suspension in PBS and flow cytometric analysis.

2.5. Methods for gene expression manipulation

2.5.1. siRNA

Optimisation

Three transfection reagents were used for optimisation of siRNA lipofection: Dharmafect #1 and #2 (both Dharmacon) and Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific). 50 000 cells were seeded for siRNA transfection at day 1 and 40 000 cells were seeded for siRNA transfection at day 2 into 24-well plates. Transfections were performed using 30nM siGLO and 0.25, 0.5, 1, 1.5, 2, 2.5µl Dharmafect reagent #1 and #2, and 0.5, 1, 1,5µl RNAiMAX were tested. siRNA or lipofection reagents were combined with OptiMEM for 5 minutes and then mixed and incubated for 20 minutes. Cell count and % transfection for each condition was determined by cell counter (Beckmann Coulter) and flow cytometry, respectively.

For experiments

Cells were transfected with 30nM FlexiTube siRNA (Qiagen) 1 day after seeding. siRNA and Dharmafect reagent #1 was added to OptiMEM in separate tubes, incubated for 5 minutes and then mixed together and incubated for 20 minutes at room temperature prior to adding to cells. RNA was collected at timepoints where cell counts/ ratios were assessed to ensure effective knockdown.

2.5.2. shRNA

shRNA cloning

pGIPZ vectors containing shRNAs were ordered from GIPZ lentiviral shRNA library (GE Dharmacon; Appendix Table 1). To clone shRNA oligos into the MSCV-miRE vector (kindly gifted by Johannes Zuber; IMP, Vienna, Austria), shRNAs were amplified from 500ng of pGIPZ vector by PCR using primers flanked with XhoI or EcoRI restriction sites (Appendix Table 2) using Platinum Pfx DNA polymerase kit (Invitrogen). Following gel purification and extraction (QIAquick gel extraction kit, Qiagen), amplified oligos and 5µg of MSCV-miRE vector were digested with XhoI and EcoRI for 3 hours, subjected to column purification (QIAquick PCR purification kit, Qiagen) and target vector was treated with alkaline phosphatase for 1 hour (NEB). Vector and oligos were ligated overnight at 16°C using T4 DNA ligase (NEB) at a molar ratio of 1:3 vector: oligo and transformed into competent DH5α bacteria (Thermo Fisher Scientific). Bacteria were spread onto ampicillin LB agar plates and

grown overnight. Colonies were picked, grown up in LB broth containing ampicillin (Sigma) and plasmid DNA was extracted and purified using CompactPrep Plasmid Midi Kit (Qiagen). Sequencing was performed to ensure correct insert had been transferred prior to infections.

Virus infection

HEK293T cells were used as packaging lines. At day 1, 20μg lentivirus (pGIPZ, lentiCRISPRv2) was transfected into 90% confluent HEK293T cells with 10μg psPAX2 and 2.5μg VSV-G using polyethylenemine (PEI). 20μg retrovirus (MSCV) was transfected with 2.5μg VSV-G using PEI. At day 2, 3×10^5 ESCs were seeded into each well of a 6-well plate. At day 3, the media from HEK293T cells was collected, passed through 0.45μm cellulose acetate filters (GE Healthcare), and incubated with ESCs cells with 4μg/ml polybrene. Two 4 hour rounds of lentiviral infections and three 3 hour rounds of retroviral infections were performed. At day 4 ESC media was changed and at day 5 2μg/ml puromycin was added to select for stably infected cells. Experiments were performed using selected cells of low passage numbers to ensure silencing of viral DNA was minimised.

2.5.3. CRISPR

Generation of 4n-Cas9 cells

4n cells were infected with lenti-Cas9-Blast (gifted from Feng Zhang, Addgene plasmid #52962; as described in *Virus infection*). Stable integrants were selected using blasticidin treatment at 2ug/ml. Cells were seeded at single-cell confluency and selected clones were screened for Cas9 expression by Western blot using anti-FLAG antibodies.

Generation of $Tsc2^{-/-}$ 4n cells

Initially 6 gRNAs were designed to target *Tsc2* exon numbers 2, 3 or 36 (Appendix Table 1). GFP-targeting gRNA was used as a control. These were cloned into vector pU6-sgRNA-EF1alpha-hygro. The pU6-sgRNA EF1Alpha-puro-T2A-BFP was a gift from Jonathan Weissman (Addgene plasmid #60955), and modified to hygro by Matias de Vas, Imperial College London. The Cas9-expressing 4n cells were infected with this vector as described (See *Virus infection*). Cells were selected with hygromycin (150ug/ml), seeded at single cell confluency, and clones were screened for loss of TSC2 by Western blot.

Targeting Tsc2 in wild-type cells

Tsc2$^{-/-}$ cells were generated by lipofection of gRNAs and Cas9 plasmids. gRNAs (Appendix Table 1) targeting the GAP domain of TSC2 were designed and cloned into a gRNA cloning vector (a gift from George Church; Addgene number #41824). Lipofection was carried out by combining 0.66μg hCas9; 0.66μg Tsc2 gRNA #1; 0.66μg Tsc2 gRNA #2; 0.125μg puromycin selection plasmid in OptiMEM and combining with 11μl Lipofectamine 2000 in OptiMEM for 20 minutes at room temperature. Transfection mix was combined with 1×10^6 cells, incubated for 10 minutes at room temperature, and seeded at single cell density. Stable integrants were selected using puromycin (2μg/ ml), selected, and screened for loss of TSC2 by Western blot.

Targeting p53

To generate p53 knockout CRISPR lines, a p53 guide (5'-GCAGACTTTTCGCCACAGCG; designed by Marieke Aarts, Imperial College London) was cloned into lentiCRISPRv2 plasmid (gift from Feng Zhang; Addgene plasmid #52961). Viruses were introduced into cells as described (see *Virus infection*). Cells were then seeded at single cell density, selected using 2ug/ml puromycin, and plated at single cell confluency. Clones were selected, expanded and screened for loss of p53 protein by Western blot.

2.5.4. Overexpression of constitutively active S6 kinase

pRK7-HA-S6K1-F5A-E389-R3A was a kind gift from John Blenis (Schalm and Blenis, 2002; Schalm et al., 2005). The insert was cloned into pyCAG (gifted by Ian Chambers) and sequenced prior to lipofection.

Transient overexpression was performed using Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific). Specifically, 0.5μg DNA was combined with 0.5μl Plus reagent in OptiMEM and incubated for 5 minutes in OptiMEM (Thermo Fisher Scientific). Following this 1.25μl LTX was added and the mixture was incubated for 30 minutes at room temperature. The DNA-lipofectamine complexes were then added in a drop wise fashion to cells which were seeded 1 day prior at a density of 2.05×10^5 cells/ ml.

Stable expression was carried out using the same protocol as transient overexpression. However, selection was carried out using puromycin (2μg/ml) to select for cells with stable

integration of the transgene. Cells were seeded at single cell density and colonies selected, expanded and screened for expression of HA.

2.6. Statistical analysis

Statistical analysis of data was carried out using GraphPad Prism v7 software. Students t-test was used for comparisons between two data groups and multiple comparisons were corrected for using the Holm-Sidak posthoc test. Two- or three-way ANOVA was used for comparisons between more than two data groups followed by Holm-Sidak or Tukey's posthoc test. Unless otherwise indicated, error bars denote standard error of the mean (SEM). In all cases, biological replicates are used and unless specified a minimum of n=3 was performed.

Chapter 3: Senescence in early mouse embryogenesis

3.1 Introduction

3.1.1 Senescence

Cellular senescence is a form of irreversible cell-cycle arrest (Munoz-Espin and Serrano, 2014). The process was first described over 50 years ago when Hayflick and Moorhead reported that cells in culture do not proliferate indefinitely (Hayflick and Moorhead, 1961). This is now known as replicative senescence and results from the gradual loss of telomeres and ensuing activation of the DNA damage response. Since this time, many stimuli have been described to induce cell senescence including oxidative stress, DNA damage, and oncogene activation. As well as entering a quiescent state, senescent cells undergo significant transcriptional changes and secrete a variety of signalling factors through the senescence associated secretory phenotype (SASP). The roles of cell senescence are diverse (Munoz-Espin and Serrano, 2014). By preventing the propagation of oncogenic cells the process acts as a potent tumour suppressor mechanism (Serrano et al., 1997; Xue et al., 2007), while its secretory effects limit tissue damage caused by fibrosis (Krizhanovsky et al., 2008) and contribute to wound healing (Demaria et al., 2014). Recently, senescence has also been shown to have a role in fine-tuning morphogenesis during embryo development (Muñoz-Espín et al., 2013; Storer et al., 2013). Despite these benefits early on in life, senescence cells accumulate with age and contribute detrimentally to age-related phenotypes. As such, efforts are now underway to clear senescence cells in order to promote healthy ageing, with promising results (Baar et al., 2017; Baker et al., 2016; Baker et al., 2011).

The mechanisms of senescence vary according to trigger, tissue, and organism (Munoz-Espin et al, 2014). However, most senescence pathways converge on cell cycle inhibitors p53, p21, or p16. These proteins act to inhibit the activity of a variety of cyclins and cyclin dependent kinases and this results in the phosphorylation and inhibition of the retinoblastoma protein. Although senescent cells often share expression of similar proteins, there is no one marker that definitively labels senescent cells. One commonly used marker is senescence-associated β-galactosidase (SAβG) which detects lysosomes. Senescent cells are marked by SAβG as an increase in autophagy and lysosomal activity occurs during senescence (Lee et al., 2006); however, β-gal staining is not limited to senescent cells. As senescent cells undergo growth arrest, loss of proliferation markers are also commonly used to identify senescent cells.

3.1.2. Senescence in development

Senescence has been shown to play a role in various tissues of mice, quail and human embryos (Muñoz-Espín et al., 2013; Storer et al., 2013). Transient structures such as the apical ectodermal ridge (AER) of the developing limb and the regressing mesonephros, a transitory embryonic kidney, stain positively for senescence markers. In these tissues, activation of the senescence program results in macrophage recruitment and clearance of cells, leading the authors to suggest that one role for developmental senescence is the elimination of transient tissues. Senescence was also observed in the developing endolymphatic sac, a structure which forms part of the ear. Different cell types contribute to the development of this structure, and the authors postulated that the activation of senescence in one cell type could permit the expansion of another cell type (Muñoz-Espín et al., 2013). Therefore, another potential role for developmental senescence could be in the regulation of the balance of different cell populations within the same developing tissue. Both studies found that developmental senescence is dependent on p21 activation, but independent of p53, p16, p15 and p19. Senescence is ablated in *p21* null embryos, although a delayed compensatory apoptosis occurs to clear the tissues which would otherwise senesce (Muñoz-Espín et al., 2013; Storer et al., 2013). This finding highlights the plasticity of early embryogenesis.

3.1.3. Senescence in competition

Senescence has been shown to play a role in the elimination of loser cells during cell competition. Studies in mice have shown that repopulating lethally irradiated bone marrow with a mixture of wild type and $p53^{+/-}$ mutant hematopoietic stem cells results in selection of cells with lower levels of p53 (Bondar and Medzhitov, 2010). Instead of being eliminated by apoptosis, loser cells with higher p53 expression show some indications of cellular senescence which could cause them to reduce their contribution to the stem cell niche. Thus, since cell senescence has previously been shown to occur during both early embryo development and cell competition, we hypothesised that the process could be relevant in mediating competitive interactions in embryonic stem cells. In this chapter, we aimed to investigate whether elimination of differentiating defective mouse embryonic stem cells occurs through activation of senescence.

3.2. Results

3.2.1. Evidence of senescence in early post-implantation embryo development but not in competition

To investigate whether loser cells are eliminated through a pathway involving senescence, co-cultures of wild-type and *Bmpr1a*$^{-/-}$ cells were stained for SAβG. We monitored staining over the first 3 days of cells in co-culture since elimination of cells starts to occur at around day 3 as they differentiate (Fig. 1.4). No β-gal positive cells were observed (Fig. 3.1), indicating that senescence is not a mechanism for cell elimination during competition. This experiment requires repetition with a positive control in order to ratify this result.

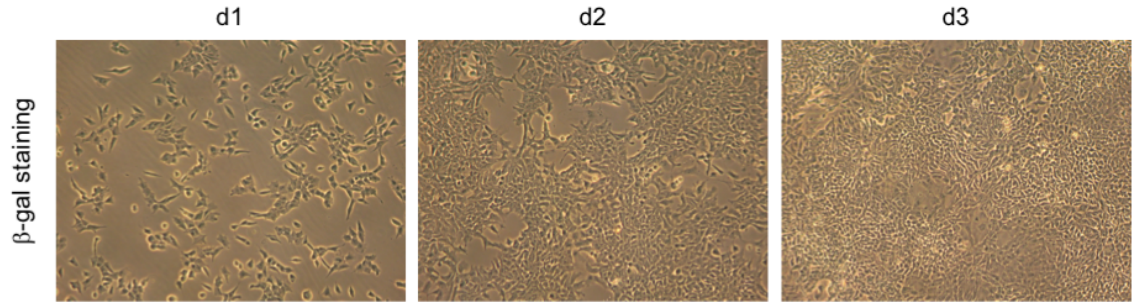


Figure 3.1: β-gal staining during competition *in vitro*.

Co-cultures of wild-type and *Bmrp1a*$^{-/-}$ cells were seeded into N2B27 media at d0 and β-gal staining was assessed over 3 days in competition. n=1.

It is possible that different processes of loser cell elimination occur *in vitro* and *in vivo*. To investigate whether senescence could be a mode of cell elimination during competition in normal development, wild-type embryos were stained for SAβG. Embryos were initially stained at embryonic day (E)12.5 in order to confirm the prior observation of senescence at later developmental stages (Muñoz-Espín et al., 2013; Storer et al., 2013). The staining, consistent with previous reports, revealed SAβG-positive cells in the apical ectodermal ridge of developing limb buds, the eyes and otic vesicles, the neural tube, and brain vesicles (Fig. 3.2A).

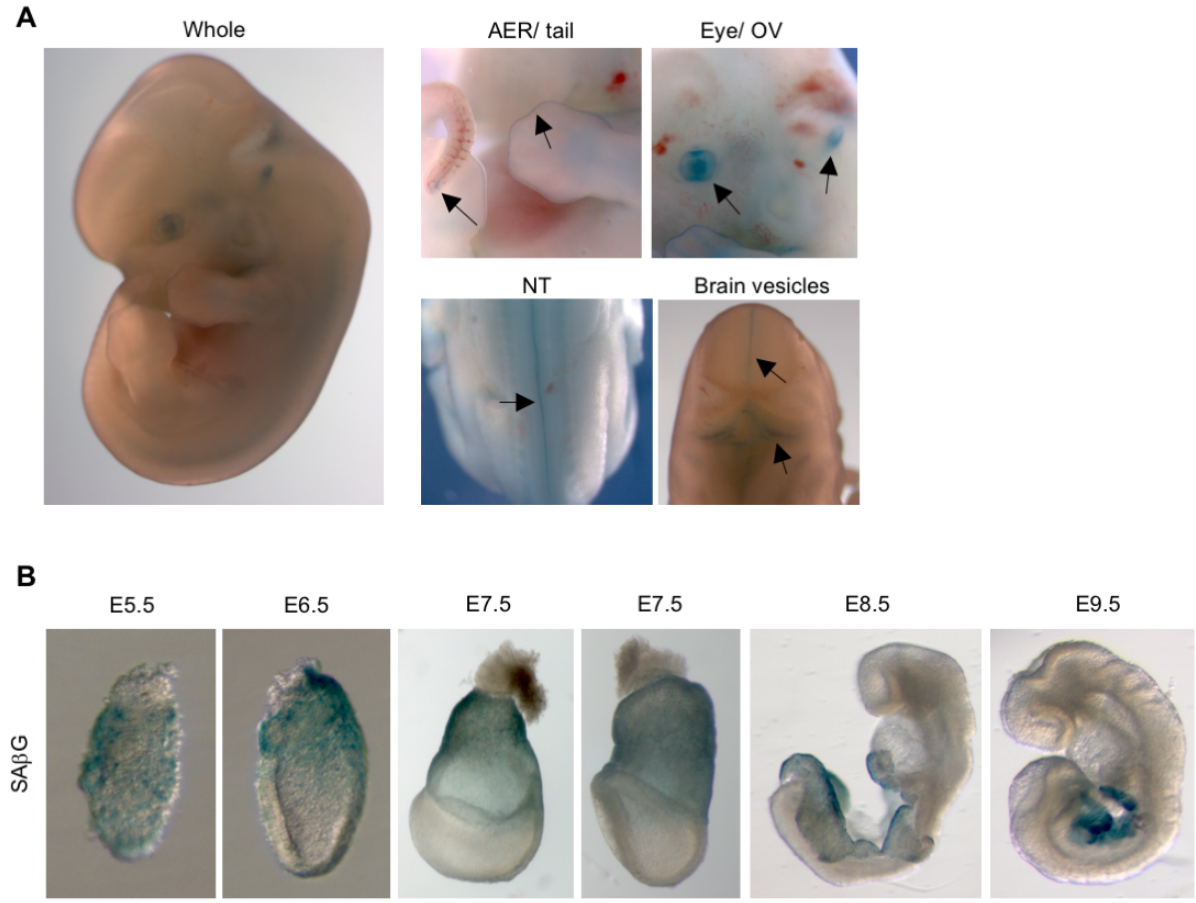


Figure 3.2: β-gal staining during early mouse development.

A. Whole E12.5 embryos were stained for SAβG to confirm previous reports of senescence in the apical ectodermal ridge (AER), tail, eye, otic vesicle (OV), neural tube (NT) and brain vesicles. B. Mice embryos from the early post-implantation E5.5 stage to E9.5 were stained for SA-β-gal. Images are reflective of staining from whole litters where n>4 for each stage.

Subsequently, wild-type embryos from the early post-implantation stages to post-gastrulation stages were dissected and stained for SAβG (Fig. 3.2B). Two observations were made. First, there was no staining of SAβG in the embryonic portion of the embryos at the stages of cell competition, indicating that senescence is not a mode of cell elimination during embryonic stem cell competition *in vivo*. Second, and interestingly, SAβG staining was observed in extra-embryonic tissues of the embryo. Specifically, at E5.5, the membrane surrounding the embryo, the visceral endoderm, stained positive for β-gal. At E6.5 and E7.5, the SAβG was restricted to the extra-embryonic visceral endoderm (ExE VE). At stages E8.5-9.5, remnants of the yolk sac, to which the extra-embryonic VE gives rise, stained for SAβG. These results suggest that although senescence does not have a role in cell competition in the epiblast cells of the embryo,

it could be activated in the extra-embryonic tissues of developing embryos where it may have other roles.

3.2.2. Reduced proliferation and upregulation of senescent markers in the extraembryonic region of early post-implantation embryos

To assess whether senescence could indeed be occurring in the extra-embryonic VE, we performed immunofluorescence staining for other markers of senescence. Another hallmark of senescence is growth arrest (Munoz-Espin and Serrano, 2014). To identify growth rates in the ExE VE, we stained E6.5 and E7.5 embryos for proliferation marker Ki67. While cells in the epiblast region of the embryo stained positively for Ki67, cells in the ExE VE stained negatively (Fig. 3.3A-C). These results suggest that the cells of the ExE VE which stain positively for SAβG are not actively dividing, further supporting a role for senescence in these cells.

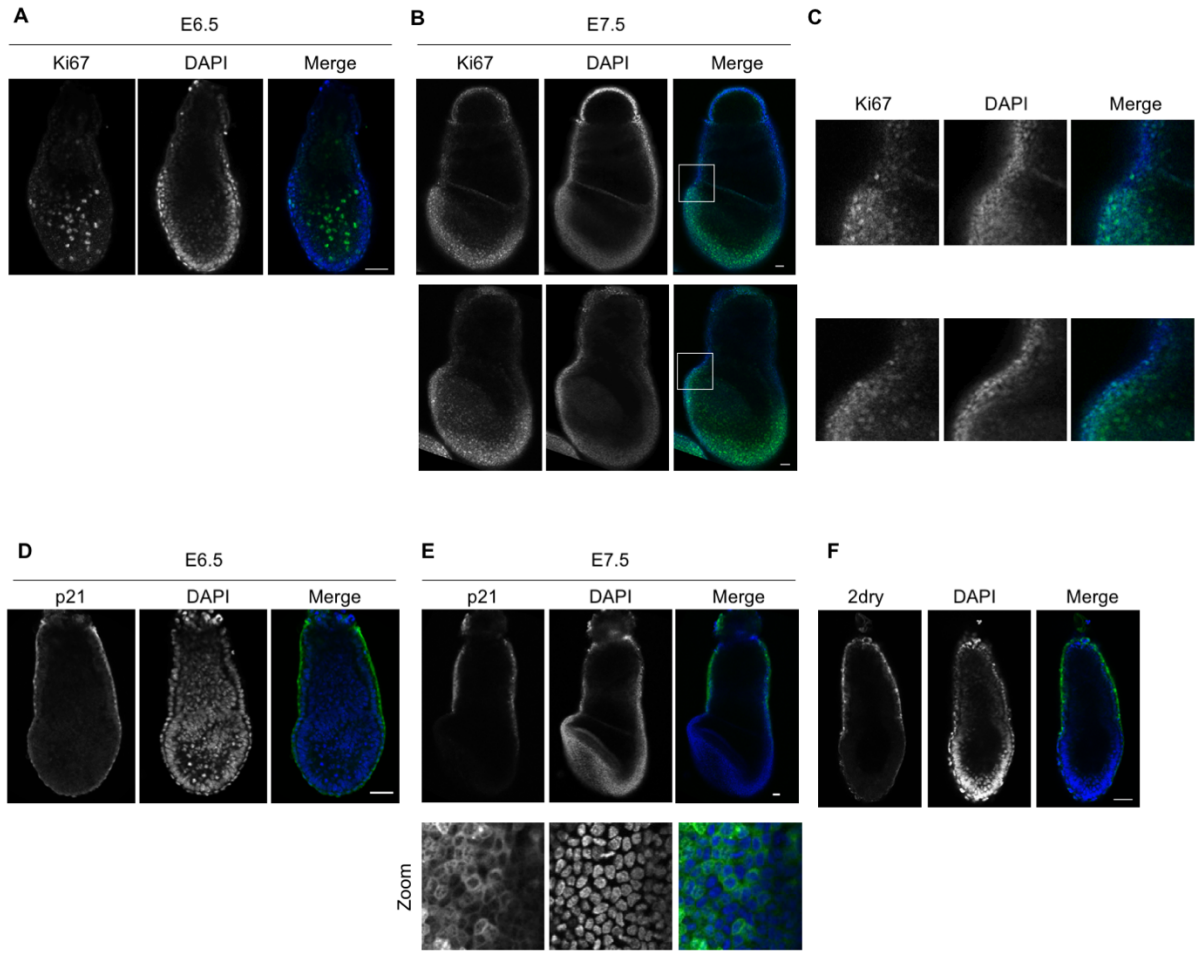


Figure 3.3: Analysis of senescence markers during early-post-implantation mouse development.

A. E6.5 mouse embryo stained for proliferation marker Ki67. B. Two E7.5 mouse embryos stained for proliferation marker Ki67. C. Zoom of E7.5 showing Ki67 staining in extra-embryonic portion (top) and embryonic portion (bottom). D. E6.5 mouse embryo stained for senescence marker p21. E. E7.5 embryos stained for senescence marker p21. Zoom shows p21 stained at surface of embryo. F. E6.5 embryos stained for secondary (2dry) antibody only. Images are reflective of staining from whole litters where n>4 for each stage.

At later stages in development, senescence is dependent on p21 (Muñoz-Espín et al., 2013; Storer et al., 2013). To assess whether senescence in the ExE VE at this earlier stage in embryogenesis could be driven by p21, we assessed expression levels of the protein by immunofluorescence. Although we observed high levels of staining in the same areas which stained positive for SAβG (Fig. 3.3D, E), this was not nuclear, indicating that the stain was non-specific. Confirming this, staining of embryos with only secondary antibodies revealed identical staining patterns (Fig. 3.3F). Therefore, we cannot conclude from our staining whether *p21* is expressed in the ExE VE, although the lack of nuclear stain suggests that the protein is not active in these cells.

We next aimed to test whether senescence genes are upregulated in the ExE VE compared to the embryonic region of embryos. For this we cut E7.5 embryos at the embryonic/ extra-embryonic boundary (Fig. 3.4A), extracted mRNA from each portion, and interrogated expression levels of several senescence genes. Control markers for the embryonic tissues (Oct4) and the for extra-embryonic tissues (HNF4A) revealed clean segregation of tissues using this method (Fig. 3.4B). Analysis of senescence markers revealed that although variability was high between three repeats, expression of *p16* was increased in the extra-embryonic portion of the embryo with respect to the embryonic portion (P=0.05). Similarly, markers *p19/ARF*, *p15*, and SASP protein *BMP4* had increased, although non-statistically significant, levels in the extra-embryonic portion of the embryo. In contrast, and consistent with the non-nuclear immunofluorescence staining, expression of p21 was not increased.

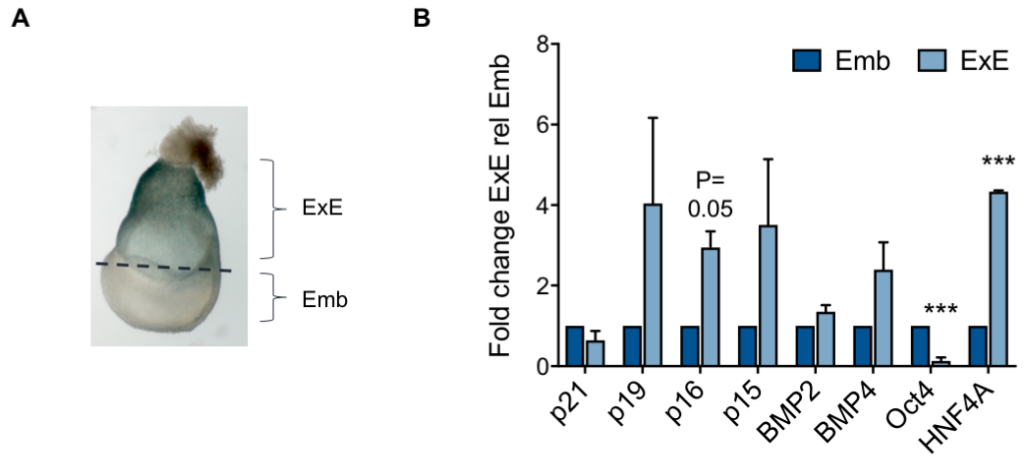


Figure 3.4: Upregulation of senescence genes in extra-embryonic portions of the embryo.

A. E7.5 embryos were cut at the extra-embryonic (ExE)/ embryonic (Emb) boundary and mRNA from pooled litters was extracted from each tissue. B. Fold change of: senescence genes p19, p16, p15, p21; SASP genes BMP2, BMP4; embryonic gene Oct4; and ExE gene HNF4A in ExE tissues relative to embryonic tissues, normalised to β-actin. Error bars denote SEM, calculated from three independent experiments which each contained a pool of ~10 embryos. ***, P<0.005, calculated by unpaired two-tailed t-test; multiple comparisons were corrected for using the Holm-Sidak method.

3.2.3. No change in β-galactosidase staining in *p53*$^{-/-}$ embryos

Since we observed no increase in the expression of p21 in the ExE VE, we analysed whether another senescence gene, p53, could be involved in driving senescence in the cells of this tissue. However, SAβG staining was identical in *p53* heterozygous and homozygous null embryos compared with wild-type embryos (Fig. 3.5), suggesting that this senescence phenotype is independent of p53 activity.

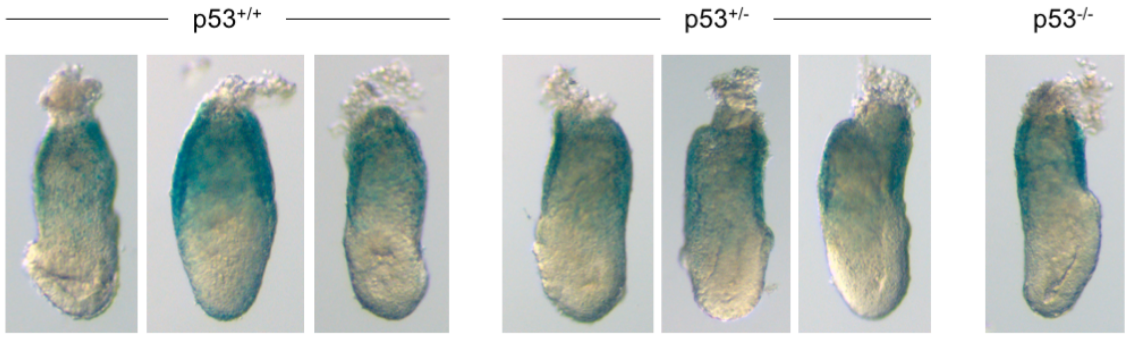


Figure 3.5: Senescence is not reduced in *p53*$^{-/-}$ embryos.

E6.5 embryos with wild-type, heterozygous, or absent expression of p53 were stained for SAβG. Genotype was confirmed by PCR.

3.3. Discussion

3.3.1. Cell senescence in development

Following on from other studies suggesting a role for senescence in both cell competition (Bondar and Medzhitov, 2010), and embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013), we sought to investigate whether senescence is involved in cell competition in the early post-implantation epiblast. As we did not identify senescence markers in the epiblast area of the embryo nor in *in vitro* culture of winner and loser cells, our results suggest that senescence is not relevant to competition. However, our results have suggested that cell senescence pathways are activated in the extra-embryonic visceral endoderm (ExE VE) of the early post-implantation embryo. This is supported by SAβG staining in the visceral endoderm, a loss of Ki67 proliferation staining in the extra-embryonic VE, and an upregulation of senescence genes in the extra-embryonic portion of the embryo compared with the embryonic area.

3.3.2. Cell senescence in the extra-embryonic VE

Although our results suggest that senescence occurs in the ExE VE, further tests will be required to fully understand if this is the case. This is because the senescence markers we have used do not exclusively mark senescent cells. Notably, SAβG reacts with lysosomal enzyme β-galactosidase and so marks any cells with high lysosomal activity (Debacq-Chainiaux et al., 2009; Lee et al., 2006). Lysosomal activity is known to be highly upregulated in the cells of the visceral endoderm (Kawamura et al., 2012), therefore there is a possibility that this high lysosomal activity is responsible for SAβG staining, rather than an induction of senescence.

Since we carried out our investigations, other researchers have investigated senescence in the early mouse embryo and reported positive staining for SAβG in the extra-embryonic VE (Huang and Rivera-Pérez, 2014). However, the authors of this study concluded that the SAβG staining is not an indication of cell senescence but results from the high lysosomal activity of these cells. This conclusion was made after *in situ* hybridisation was used to analyse the expression of p21 and no staining was observed in the areas of the embryo which stained positively for SAβG. Furthermore, the authors stained embryos for mitosis marker phospho-histone H3 to identify cells which are actively dividing. As cells in the extra-embryonic VE stained positively for phospho-histone H3, it was concluded that these cells are not undergoing a cell-cycle arrest and so are not senescent. However, it was reported that only a small fraction

of cells in the extra-embryonic endoderm stained positively for phospho-histone H3 (2.06% of all extra-embryonic VE cells; Huang and Rivera-Pérez, 2014). Similarly, we have shown that a significant proportion of extra-embryonic VE cells do not stain for Ki67, a marker of active cell cycling, while staining is observed in large portions of the embryonic region. Future investigation would benefit from bromodeoxyuridine (BrdU) staining which would allow a more dynamic assessment of whether cells of this region are actively dividing.

Previous studies investigating developmental senescence have revealed that the process is dependent on p21, as loss of developmental senescence at E12.5 onwards occurs in *p21*-null embryos (Muñoz-Espín et al., 2013; Storer et al., 2013). Cell cycle regulators p16 and p19 are not involved in cell senescence at this later stage in mouse development. In contrast, our data, consistent with others, has revealed that p21 is not upregulated in the extra-embryonic tissues of the embryo which stain for SAβG (Huang and Rivera-Pérez, 2014). Furthermore, we did not observe a change in SAβG when the p21 regulator, *p53*, was deleted. Together, these results suggest that if senescence does occur during early post-implantation development, the mechanism is *p21*-independent and therefore different to that which is activated at later stages of development. Instead, we have observed a trend for upregulation of p19, p15 and p16 in the extra-embryonic tissues, suggesting that senescence-associated proliferative arrest in this early developmental context may be more dependent on these cell cycle inhibitors rather than p53 or p21. To test this, it will be important to assess whether embryos lacking *p16/p19ARF* have reduced SAβG staining of the extra-embryonic VE.

3.3.3. Potential roles of cell senescence in the extra-embryonic VE

Although further work is required to validate the presence of senescence in the ExE VE, our data thus far support the hypothesis that senescence is activated in this tissue. The VE surrounds the extra-embryonic and epiblast region of the developing embryo at E5.5. At the formation of the primitive streak, the anterior VE has important roles in signalling to the underlying epiblast and instructing cell fate (Beddington and Robertson, 1999; Perea-Gomez et al., 2002). As gastrulation occurs, the visceral endoderm largely becomes restricted to the extra-embryonic portion of the embryo as the definitive endoderm is formed at the anterior tip of the embryo (Stern, 2008). There are various potential roles for senescence in the VE. Firstly, senescence could mediate a growth arrest that would allow replacement of anterior VE cells by the definitive endoderm. Secondly, senescence could have a role in autocrine lineage specification

of the ExE VE fate. This possibility is supported by our qPCR data revealing increased expression of SASP component BMP4, a signalling molecule that patterns the VE (Paca et al., 2012). Finally, it is possible that through the SASP, senescence could allow paracrine signalling to the underlying epiblast or extra-embryonic tissues to modulate cellular differentiation or patterning.

Chapter 4: The mTOR pathway is an effector of cell competition during early mammalian development

4.1. Introduction

4.1.1. The mTOR pathway

Mechanistic target of rapamycin (mTOR) is a serine/ threonine protein kinase which forms the catalytic subunit of two separate protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 integrates signals from a wide variety of external inputs, including oxygen, amino acids and energy status, and is a master regulator of cellular metabolism and growth (Saxton and Sabatini, 2017). In a positive growth environment, the complex activates anabolic pathways and represses catabolic ones. As such, mTOR has an integral role in the homeostatic control of cell activity by coupling a wide range of intrinsic and extrinsic cues with the large-scale alteration of cellular behaviour. Much less is known about mTORC2, although the complex is involved in the regulation of cell proliferation, survival, and migration (Saxton and Sabatini, 2017). Unless otherwise stated, I will refer to mTORC1 as simply 'mTOR' as I am primarily investigating the activity of the first complex.

4.1.2. Regulation of mTOR

The mTOR complex is active at the lysosomal membrane, and two main upstream regulatory pathways mediate its activity by controlling either its cellular localisation or its activity at the lysosome.

Tuberous sclerosis complex

One key regulatory input into mTOR comes from the tuberous sclerosis complex (TSC) which is composed of TSC1, TSC2 and TBC1D7 and functions as an inhibitor of mTOR activity (Dibble et al., 2012; Inoki et al., 2003b). The complex acts as a GTPase activating protein (GAP) for target Rheb, a small GTPase which is located at the lysosomal membrane (Inoki et al., 2003a; Tee et al., 2003). When GTP-bound, Rheb activates mTOR through a poorly resolved enzymatic process, and when GDP-bound, mTOR remains inactive (Long et al., 2005; Sancak et al., 2007). By catalysing the hydrolysis of GTP to GDP, TSC represses Rheb, and therefore mTOR, activity. TSC activity is regulated largely post-translationally downstream of a wide range of upstream signals, including the insulin and growth factor signalling pathways (Inoki et al., 2002; Inoki et al., 2006; Lee et al., 2007; Ma et al., 2005; Manning et al., 2002;

Menon et al., 2014; Roux et al., 2004), energy status (Inoki et al., 2006; Inoki et al., 2003b), oxygen (Brugarolas et al., 2004; DeYoung et al., 2008), DNA damage (Budanov and Karin, 2008; Feng et al., 2007a) and a range of other stresses (Demetriades et al., 2016).

Rag GTPases

The other major signalling input into mTOR is the RagGTPases, which sense amino acid availability. These proteins modulate mTOR activity by tethering the mTOR complex to the lysosomal membrane, where it can be activated by Rheb (Sancak et al., 2010). In recent years it has emerged that the activity of RagGTPases is controlled by sets of proteins with GAP, GEF (GTP-exchange factor) or GDI (GTP-dissociation inhibitor) activity which have remarkable specificity for the individual amino acids which they sense (Bar-Peled et al., 2013; Chantranupong et al., 2016; Chantranupong et al., 2014; Jung et al., 2015; Peng et al., 2017; Rebsamen et al., 2015; Saxton et al., 2016; Wang et al., 2015; Wolfson et al., 2016; Wolfson et al., 2017). In addition, glucose metabolism can act through the RagGTPases to control mTORC1 signalling (Efeyan et al., 2013; Kalender et al., 2010).

4.1.3. Downstream of mTOR

Pathways activated downstream of mTOR function to increase resources required for cell growth. This is mediated by mTOR through the regulation of protein synthesis, protein degradation, and lipid, nucleotide and glucose metabolism.

Protein synthesis

mTOR promotes protein synthesis largely through phosphorylation of its effectors 4E-BP and S6 kinase. 4E-BP acts as inhibitor of translation by sequestering translation initiation factor protein eIF4E . Upon mTOR-mediated phosphorylation, 4E-BP activity is uninhibited and 5-cap dependent mRNA translation proceeds (Brunn et al., 1997; Gingras et al., 1999). In particular, phosphorylation of 4E-BP results in the translation of mRNAs containing 5' TOP motifs which encode proteins of the translational machinery (Hsieh et al., 2012; Thoreen et al., 2012). S6 kinase phosphorylates various proteins involved in protein synthesis, including the ribosomal protein S6 (S6), and eukaryotic translation initiation factor 4B (eIF4B). The cellular function of S6 remains elusive. $S6^{P-/-}$ knockin mice, which contain mutated non-phosphorylatable residues at five S6 phosphorylation sites, exhibit altered glucose metabolism and reduced size of several cell types (Ruvinsky et al., 2005), while ablation of the protein in

mice livers results in proliferation defects following hepatectomy (Volarevic et al., 2000). In contrast, complete S6 ablation results in mouse embryo lethality at gastrulation stages (Panić et al., 2006). Therefore, although the gene is essential for proper development, its roles in the adult mouse are more subtle but may be important for cell size and proliferation control.

Protein degradation

The mTOR pathway also instructs activity of several pathways involved in protein turnover, including autophagy, lysosome biogenesis, and proteasome activity. Autophagy is a self-degradation system which targets used organelles for destruction and recycling (Kaur and Debnath, 2015). Cellular components targeted for degradation through the autophagy pathway are engulfed by vesicles called autophagosomes, which fuse with acidic lysosomes to digest internal material. Autophagy plays a key role in protein homeostasis, but is also upregulated in times of stress to mobilise energy stores. When the mTOR pathway is active, autophagy is inhibited directly through the phosphorylation and inhibition of several autophagy proteins such as unc-51-like kinase 1 (ULK1; Kim et al., 2011). mTOR activation also results the disruption of lysosome biogenesis through phosphorylation and inhibition of transcription factor EB (TFEB), a master regulator of lysosomal gene expression (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Consequently, in response to starvation, mTOR repression and autophagy activation allows increased recycling of cellular components. Finally, mTOR blocks proteasome-dependent protein degradation through modulating ubiquitination (Zhao et al., 2015) and levels of proteasomal chaperones (Rousseau and Bertolotti, 2016).

Lipid, nucleotide and glucose metabolism

In order for growth to occur, cells must produce lipids for nascent membrane formation, nucleotides for DNA replication, and must intake glucose for metabolism. mTOR promotes lipid production through activation of transcription factor sterol responsive element binding protein (SREBP1), which regulates expression of genes involved in lipid synthesis (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2008). Synthesis of nucleotides occurs through two pathways: mTOR-mediated S6K1 phosphorylation triggers the activation of carbomoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase (CAD), an enzyme involved in pyrimidine synthesis (Ben-Sahra et al., 2013), while purine synthesis is activated through phosphorylation of ATF4 and subsequent induction of methylenetetrahydrofolate

dehydrogenase 2 (MTHFD2) expression (Ben-Sahra et al., 2016; French et al., 2016). Finally, glycolysis is promoted through activation of HIF1α (Duvel et al., 2010).

4.1.4. mTOR in development

Proper mTOR activity is crucial for development: deletion of the protein in mice results in embryonic lethality at gastrulation stages (Murakami et al., 2004). Similarly, mice which are unable to detect amino acid availability as a result of constitutive activation of RagA die soon after birth (Efeyan et al., 2013), while mice lacking *Tsc2* fail to survive past E9.5 (Onda et al., 1999). In culture, an upregulation of mTOR activity occurs at the onset of ESC differentiation (Cherepkova et al., 2016), and it has been demonstrated that this upregulation is necessary for differentiation, since knockdown of Tsc2 maintains cells in the pluripotent state (Betschinger et al., 2013).

In this chapter we aimed to investigate mechanisms important for cell competition during gastrulation. Our approach was to delineate which pathways had altered activity in *Bmpr1a*$^{-/-}$ cells in competition versus separate culture conditions by transcriptomic analysis.

4.2. Results

4.2.1. Assessment of mTOR levels during competition

IPA analysis of microarray data reveals alteration of mTOR-related gene sets in competition

Prior to my arrival in the lab, a microarray had been performed to compare gene signatures of wild-type and *Bmrp1a*$^{-/-}$ cells in separate and co-culture. The experiment, as outlined in Fig. 4.1A, was carried out by culturing the cells together and separately for 3 days in N2B27, FACS sorting into each cell type, and subjecting to microarray analysis. The microarray sample preparation was carried out by Margarida Sancho. Subsequent Ingenuity Pathway Analysis (IPA) revealed a high Z-score of mTOR pathway-associated components specifically in the *Bmpr1a*$^{-/-}$ cells during competition (Fig. 4.1B-C). Specifically, genes associated with mTOR itself, its target S6 kinase, and mTOR regulatory complex LAMTOR, were downregulated in co-culture in *Bmpr1a*$^{-/-}$ cells with respect to wild-type cells (Fig. 4.1C). Conversely, genes associated with downregulation of mTOR, such as those associated with mTOR inhibitor everolimus treatment and lysosomal activator TFEB activation, were found to be upregulated in co-cultured *Bmpr1a*$^{-/-}$ with respect to wild-type cells (Fig. 4.1C). Gene changes associated with the mTOR pathway are detected due to the control of transcription factors such as TFEB and SREBP1 downstream of mTOR activation (Saxton and Sabatini, 2017).

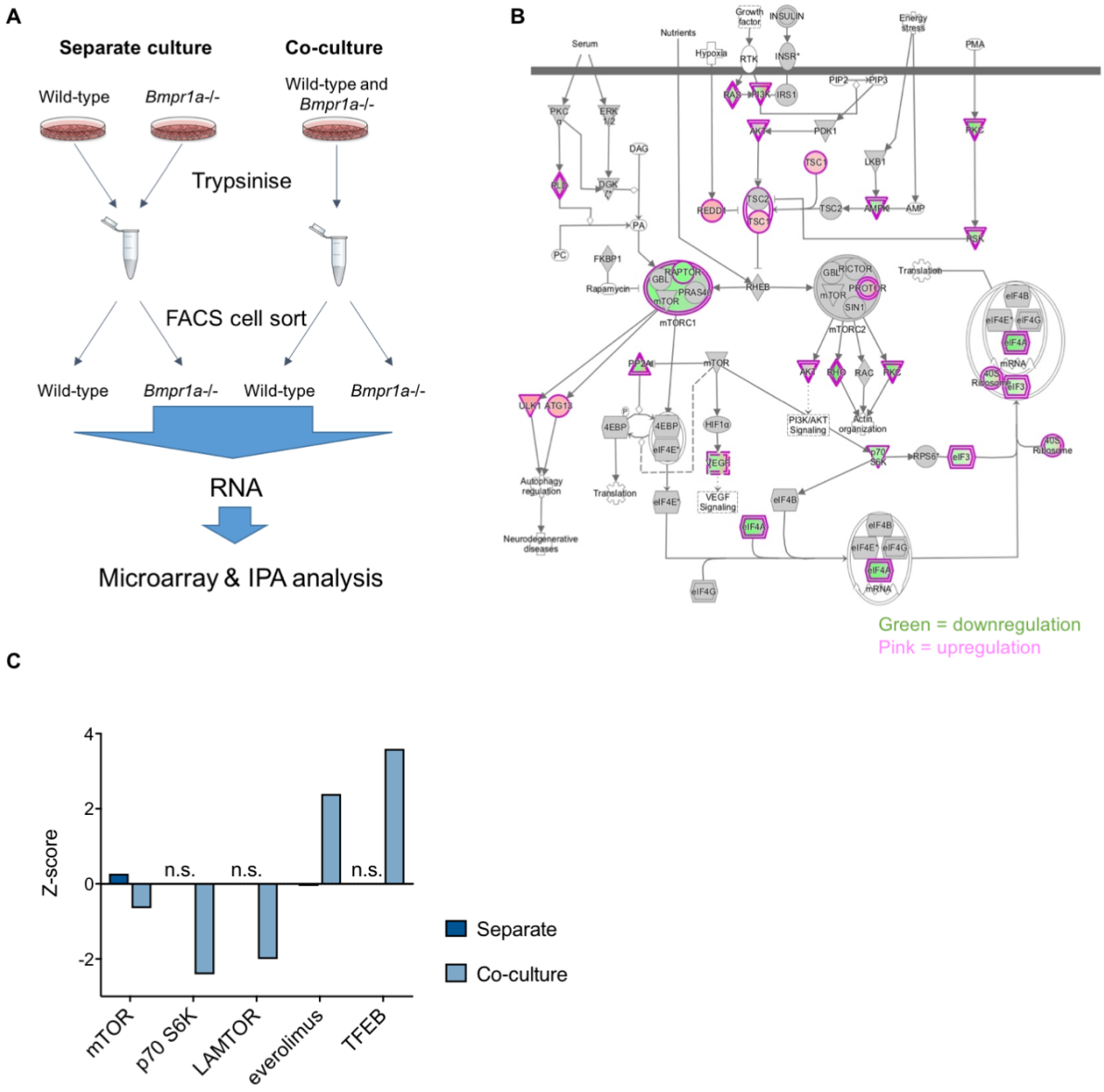


Figure 4.1: Gene expression profiling indicates downregulation of genes associated with the mTOR pathway in *Bmpr1a*$^{-/-}$ cells during cell competition.

A. Experimental setup. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured together and separately for three days in N2B27 media, trypsinised, sorted by FACS, and the mRNA of each cell type was subjected to microarray and IPA analysis. B. IPA heatmap depicting pathways up- (pink) or down- (green) regulated in *Bmpr1a*$^{-/-}$ compared with wild-type cells during competition. C. Z-score, which indicates fold-change of pathway genes, of several mTOR-associated gene sets in *Bmpr1a*$^{-/-}$ compared with wild-type cells in separate culture and co-culture. n.s., not significant. Microarray sample preparation was performed by Margarida Sancho (Imperial College London).

mTOR activity is reduced in Bmpr1a$^{-/-}$ cells specifically during cell competition

To test whether the mTOR pathway is indeed differentially activated in loser and winner cells during cell competition, we performed the FACS-sort as described in Fig. 4.1A but collected protein and analysed the expression and phosphorylation status of several markers of the mTOR pathway by Western blot (Fig. 4.2A). We observed reduced phosphorylation of pS6 and also S6 kinase at T389, a site exclusively targeted by mTOR, in *Bmpr1a*$^{-/-}$ cells when cultured with wild-type cells, but not when cultured alone in separate culture. We confirmed this result by immunofluorescence (Fig. 4.2B, C). Interestingly, we also observed an upregulation of mTOR pathway components in the wild-type cells during competition compared with the separate conditions (Fig. 4.2A).

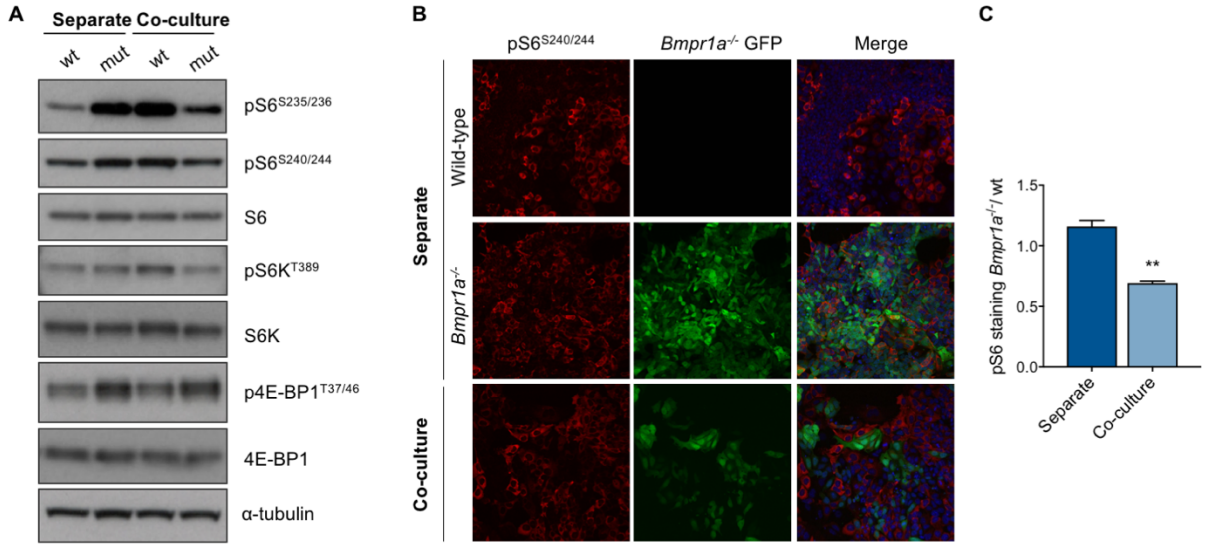


Figure 4.2: Reduced levels of mTOR pathway activity in *Bmpr1a*$^{-/-}$ cells during competition.

A. Wild-type and *Bmrp1a*$^{-/-}$ (mut) cells were cultured together and separately for 3 days in N2B27, sorted by FACS, and extracted protein lysates were immunoblotted to assess levels of several markers of the mTOR pathway. B. Wild-type and *Bmpr1a*$^{-/-}$ cells were culture together and separately for 3 days in N2B27 and levels of pS6$^{S240/244}$ were assessed by immunofluorescence. C. Quantification of pS6 levels in *Bmpr1a*$^{-/-}$ cells relative to wild-type cells. **, $p<0.01$, two-tailed t-test. Error bars denote SEM. n=3.

In order to gain a more quantitative and single-cell assessment of the changes in S6 phosphorylation, stained cells were analysed by flow cytometry. One target of the activated mTOR complex 1 is S6 kinase, which in turn phosphorylates S6 at phosphorylation sites S240/244 and S235/236. While the S235/236 site is also targeted by p90 RSK downstream of the ERK signalling pathway, the S240/244 site is exclusively phosphorylated by S6K1/2

downstream of the mTOR pathway. As we were interested in activity of the mTOR pathway, we tested the sensitivity of the $pS6^{S240/244}$ antibody. In normal conditions, most cells stained positively for pS6 (Fig. 4.3A). This staining was lost upon conditions of mTOR pathway inhibition, including rapamycin treatment and starvation (EBSS media), indicating that flow cytometry can be used to provide an accurate readout of S6 phosphorylation. Optimisation of the antibody was also carried out to identify the optimal dilution for flow cytometric analysis (Fig. 4.3B). We observed that a dilution of 1:200 led to the highest level of staining out of four tested dilutions and used this for subsequent experiments.

Next, we used flow cytometry to assess the changes in mTOR activity in the whole population of cells over the first three days of competition. Levels of pS6 were similar between cell types at day one in both separate culture and co-culture (Fig. 4.3C, D). By day two, *Bmrp1a*$^{-/-}$ cells showed a reduction in pS6 staining specifically in co-culture, and at day three the majority of loser cells had low staining. This is despite these cells sustaining a higher level of pS6 staining than wild-types in separate conditions, a difference we attribute to differences in confluency and therefore available media resources between the culture of the two cell types. In contrast, in separate culture *Bmpr1a*$^{-/-}$ cells sustained high levels of pS6 staining at day three. Furthermore, levels of total S6 remained similar between cell types at day 3 (Fig. 4.3C, right). Notably, this single-cell analysis allowed us to measure the proportion of cells which had lost pS6 staining. We found an increase in 'low pS6' cells specifically in competing defective cells at day 3 (Fig. 4.3D). Culture of cells in ESC media, where pluripotency is maintained and competition dynamics are not observed, did not lead to a reduction in pS6 staining in the loser cell population (Fig. 4.3E).

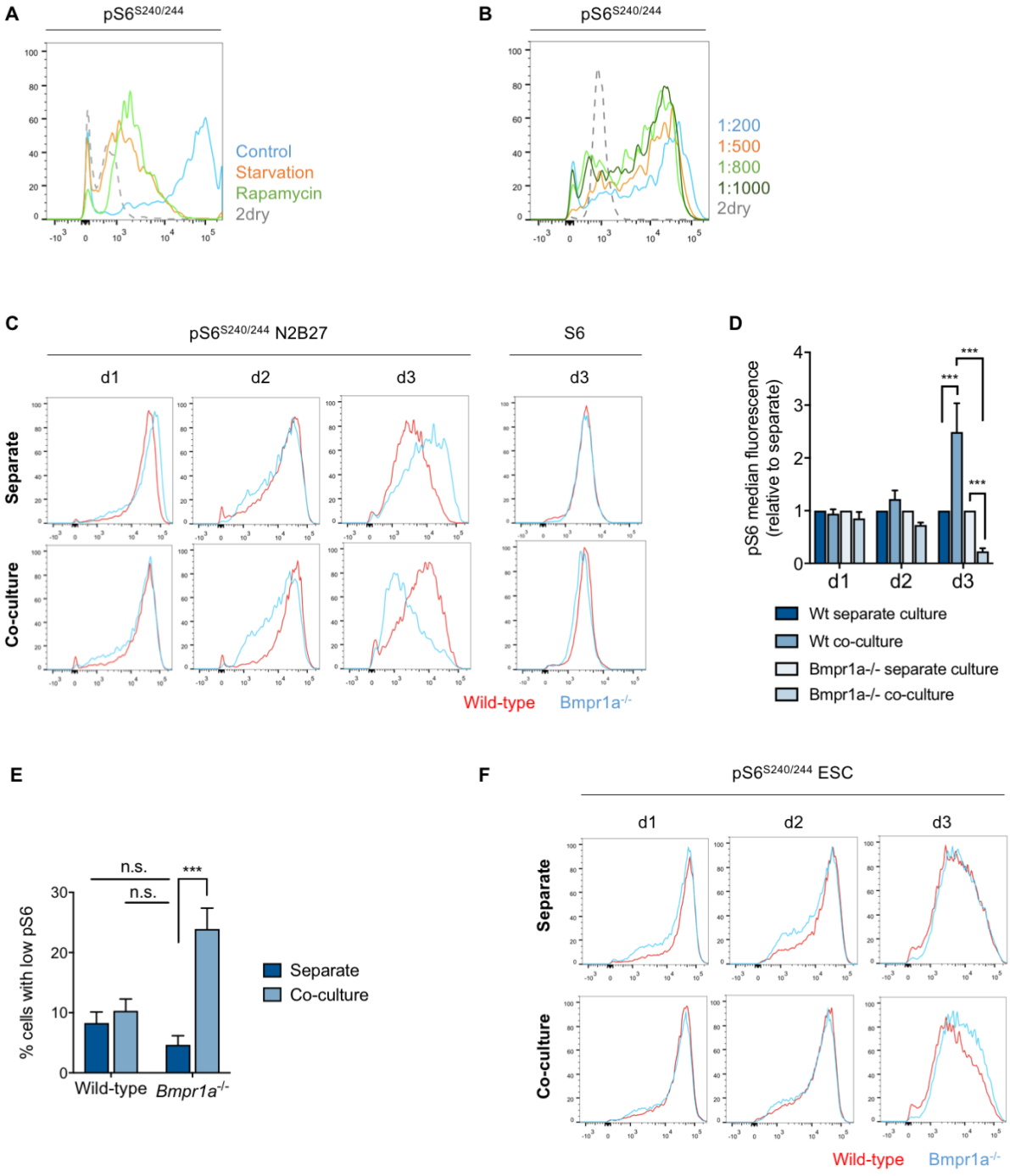


Figure 4.3: Quantitative assessment of pS6 levels by flow cytometry

A. Sensitivity of pS6$^{S240/244}$ antibody was tested by assessing pS6 levels in control, starved, or rapamycin-treated cells. B. Antibody was titrated to assess optimal dilution for further use. C. pS6$^{S240/244}$levels in *Bmpr1a*$^{-/-}$ and wild-type cells over 3 days in separate and co-culture, and total S6 levels at day 3 (right). D. Quantification of pS6$^{S240/244}$ levels in *Bmpr1a*$^{-/-}$ and wild-type cells in co-culture relative to separate culture over three days in N2B27. E. Percentage of wild-type and *Bmpr1a*$^{-/-}$ cells with low pS6$^{S240/244}$ levels in separate and co-culture. F. pS6$^{S240/244}$ in conditions where

pluripotency is maintained by serum + LIF. ***, p<0.001, 2-way ANOVA and Tukey's post-hoc test. Error bars denote SEM. n=3.

Another key downstream target of the mTOR pathway is 4E-BP1. We performed flow cytometry to gain a quantitative and single-cell assessment of p4E-BP1 levels across the whole cell population. Cells which were starved or treated with mTOR inhibitor Torin demonstrated reduced or absent p4E-BP1 staining, respectively, indicating that the antibody used was effective at conveying p4E-BP1 levels by flow cytometry (Fig. 4.4A). We did not detect reduced levels of p4EBP1 in loser cells during competition (Fig. 4.4B), consistent with our results collected by sorting cells and analysing p4E-BP1 levels by Western blot (Fig. 4.2A) suggesting that this axis downstream of mTOR is not affected during competition.

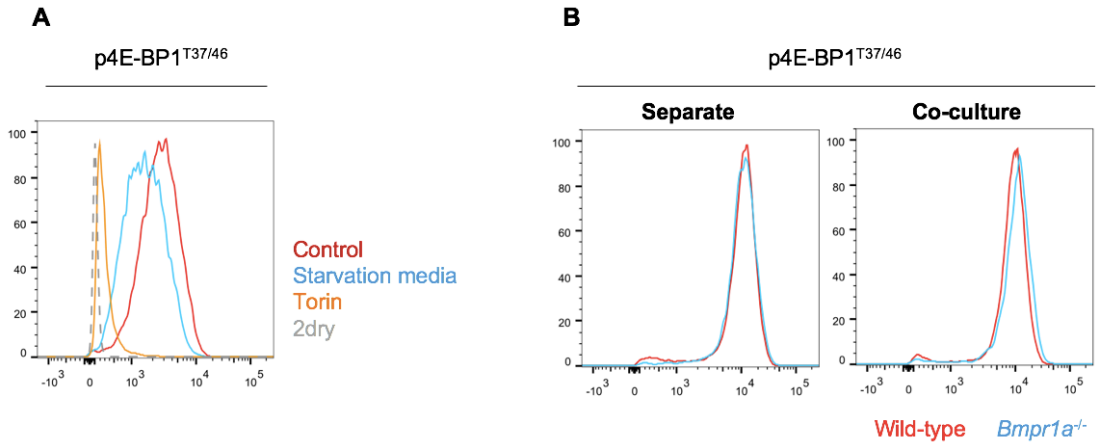


Figure 4.4: No change in p4E-BP1 levels in *Bmpr1a*$^{-/-}$ cells in co-culture

A. Sensitivity of p4E-BP1 antibody was tested by assessing p4E-BP1 levels in control, starved, and torin-treated cells. B. p4E-BP1 levels in wild-type and *Bmpr1a*$^{-/-}$ cells at day 3 in N2B27 in separate and co-culture.

mTOR activity is reduced in tetraploid cells during competition

To identify whether loss of mTOR activity in loser cells is specific to *Bmrp1a*$^{-/-}$ cells or is a general feature of cell competition, we interrogated mTOR pathway activation levels in another model of competition: the co-culture of wild-type and tetraploid (4n) cells. As with the *Bmpr1a*$^{-/-}$ competition, we observed reduction of pS6 staining in the 4n cells specifically when cultured with wild-type cells at day 3 in competition, but not when cultured alone (Fig. 4.5A-C). As before, in conditions where pluripotency was maintained, no differences in pS6 staining was observed between cells types when cultured either separately or together (Fig. 4.5B, right). Our

results suggest that during cell competition, mTOR activity is repressed in loser cell populations.

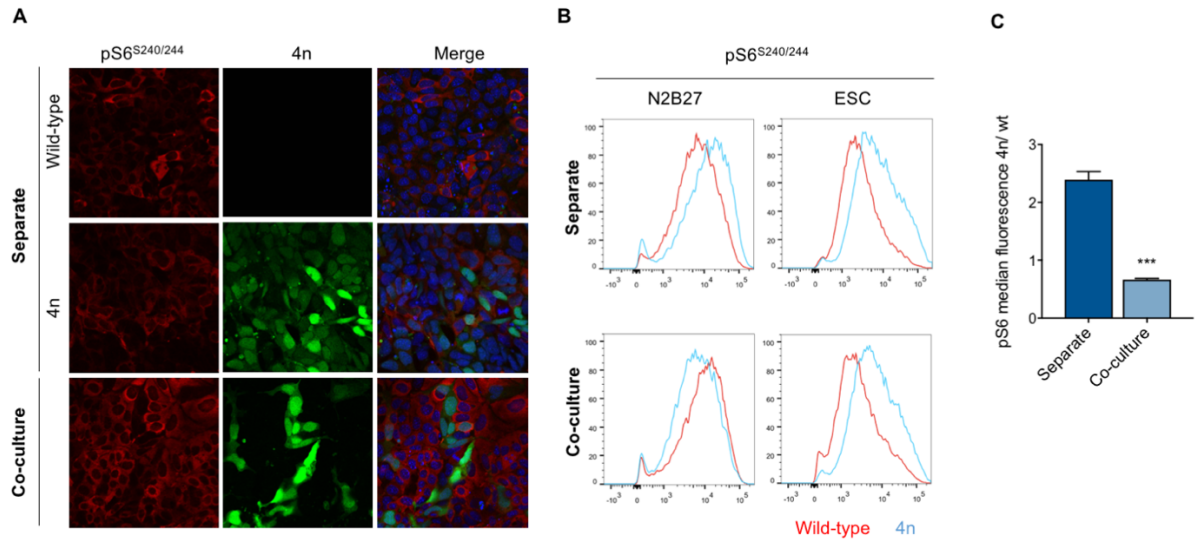


Figure 4.5: Reduced levels of mTOR pathway activity in 4n cells during competition.

A. Wild-type and 4n cells were cultured separately and together for 3 days in N2B27, fixed, and pS6 levels were assessed by immunofluorescence. B. Wild-type and 4n cells were cultured separately and together for 3 days in N2B27 (left) or ESC (right) media and pS6 levels were assessed by flow cytometry. C. Quantification of pS6 levels during differentiation in 4n cells relative to wild-type cells in separate and co-culture assessed by flow cytometry median fluorescence. ***, $p<0.001$, two-tailed t-test. Error bars denote SEM. n=3.

4.2.2. mTOR activity is required for cell viability during differentiation

It is possible that the observed reduction of mTOR activity in loser cells is a result of cell death during cell competition. To test whether this is the case, we used caspase inhibitors to prevent cell apoptosis (Fig. 4.6A). It has been previously shown that caspase inhibition rescues the elimination of loser cells in competition (Sancho et al., 2013). We found that preventing cells from dying resulted in an accumulation of cells with low pS6 staining (Fig. 4.6B,C). This suggests that loss of mTOR activation precedes death, and that cells with low mTOR activity are destined for apoptosis. To test this second idea further, we treated cells with mTOR-inhibitor rapamycin and assessed cell viability by analysing cell counts and levels of cell death markers. Rapamycin treatment reduced cell counts to around 50% of control levels, and this was rescued by caspase inhibition, suggesting that the reduced cell number is due to apoptosis rather than a cell growth arrest (Fig. 4.6D). mTOR inhibition by rapamycin also led to an increase in apoptosis markers cleaved PARP and cleaved caspase-3 (Fig. 4.6E,F). This effect

was not due to changes in mTORC2 pathway activation as levels of p-AKT^{T473} were not altered. Interestingly, cells maintained in the pluripotent state were not sensitive to mTOR inhibition (4.6E,F). We conclude that specifically during differentiation, cells become sensitised to repression of mTOR pathway activity and that this reduction in loser cells in a competitive environment is sufficient to trigger their elimination.

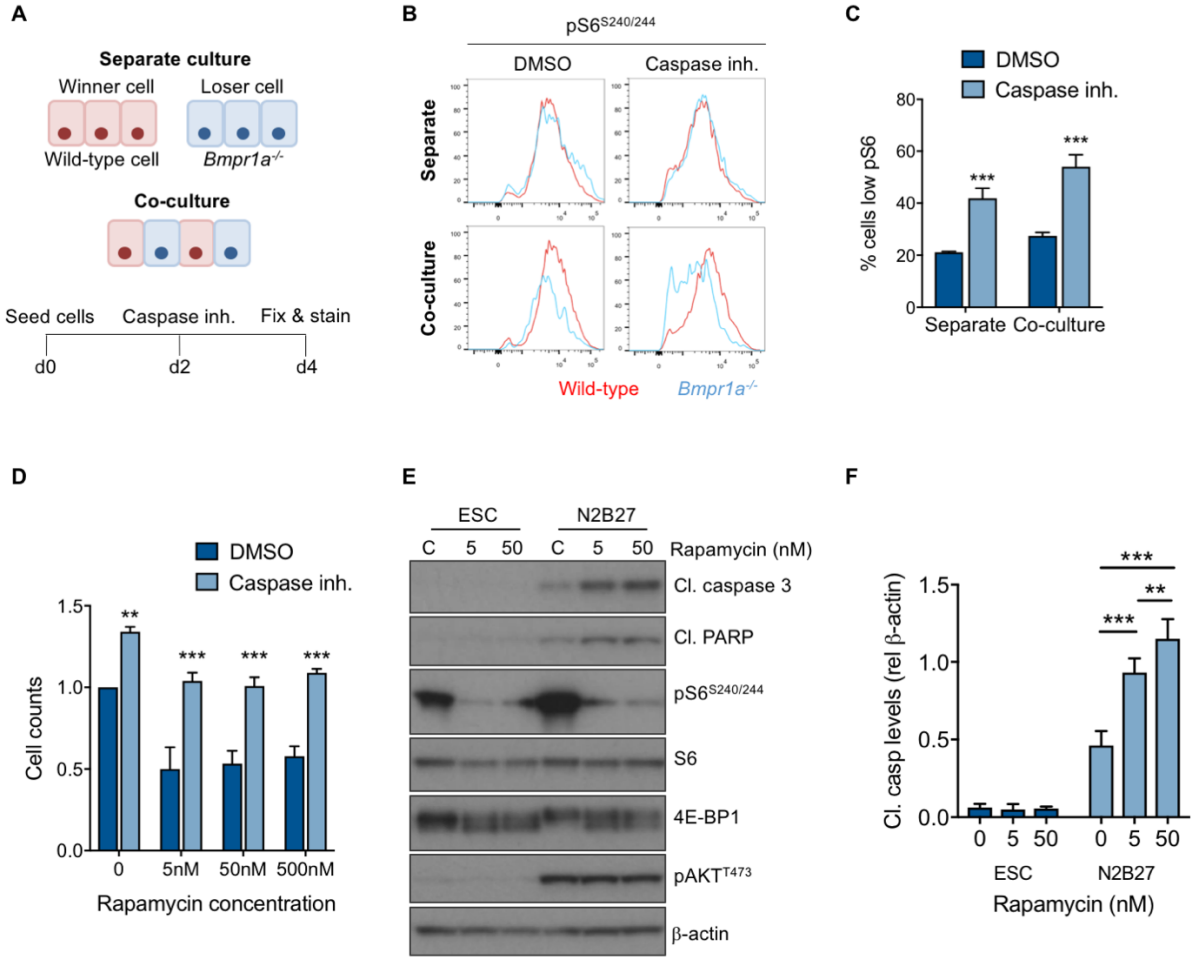


Figure 4.6: Repression of mTOR pathway activity triggers apoptosis in differentiating ESCs.

A. Cartoon depicting experimental setup. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured together and separately in N2B27. At day 2, cells were treated with 100μM Z-VAD-FMK caspase inhibitors, and at day 4, cells were fixed and stained for pS6. B. pS6 staining in wild-type and *Bmpr1a*$^{-/-}$ cells at day 4 in separate and co-culture with and without caspase inhibitors. C. Percentage of *Bmpr1a*$^{-/-}$ cells exhibiting low levels of pS6 staining at day 4 in competition following treatment with or without caspase inhibitors. D. Cell counts of wild-type cells treated with rapamycin for 24h in the presence and absence of caspase inhibitors. E. Pluripotent (ESC) and differentiating (N2B27) wild-type cells were treated with rapamycin for 6 hours and cell death markers were assessed by western blot. F. Quantification of cl. caspase-3 levels in (E) relative to β-actin. **, $p<0.01$; ***, $p<0.001$, 2-way ANOVA followed by Sidak's post-hoc test. Error bars denote SEM. n=3 for all experiments.

4.2.3. Hyperactivation of the mTOR pathway

Optimisation of siRNA protocol

We next asked if loss of mTOR pathway activation was necessary for the elimination of defective cells during competition. To answer this question we attempted to sustain high levels of mTOR pathway activity in competing cells through knockdown of upstream inhibitor TSC2. First, we targeted this protein for knockdown using siRNAs. Transfection protocols were optimised by assessing transfection efficiency (% cells positive for siGLO fluorescent siRNA) and cell viability (cell counts) of three different transfection reagents (Dharmafect #1, #2 and RNAiMAX) at two transfection time-points: 1 day after cell plating and 2 days after cell plating. Since cell competition starts to occur from 3 days after cell plating (Fig. 1.4), we aimed to achieve highest transfection efficiency and cell viability at this time-point. Transfection efficiency was highest when cells were transfected 1 day after plating compared to 2 days (Fig. 4.7 A compared with B, bottom). Cell viability was highest in cells treated with Dharmafect #1 reagent at low volumes (Fig. 4.7A,B, top). Since 0.5ul of Dharmafect #1 did not compromise cell viability but resulted in high transfection efficiency at day 1, we used this condition for further transfections.

The knockdown efficiency of four different Tsc2-targeting siRNA oligos was assessed at the protein level by Western blot and at the mRNA level by qPCR. Oligo #1 yielded greatest knockdown at the protein and mRNA level, and this oligo was used for the following experiments (Fig. 4.7C,D).

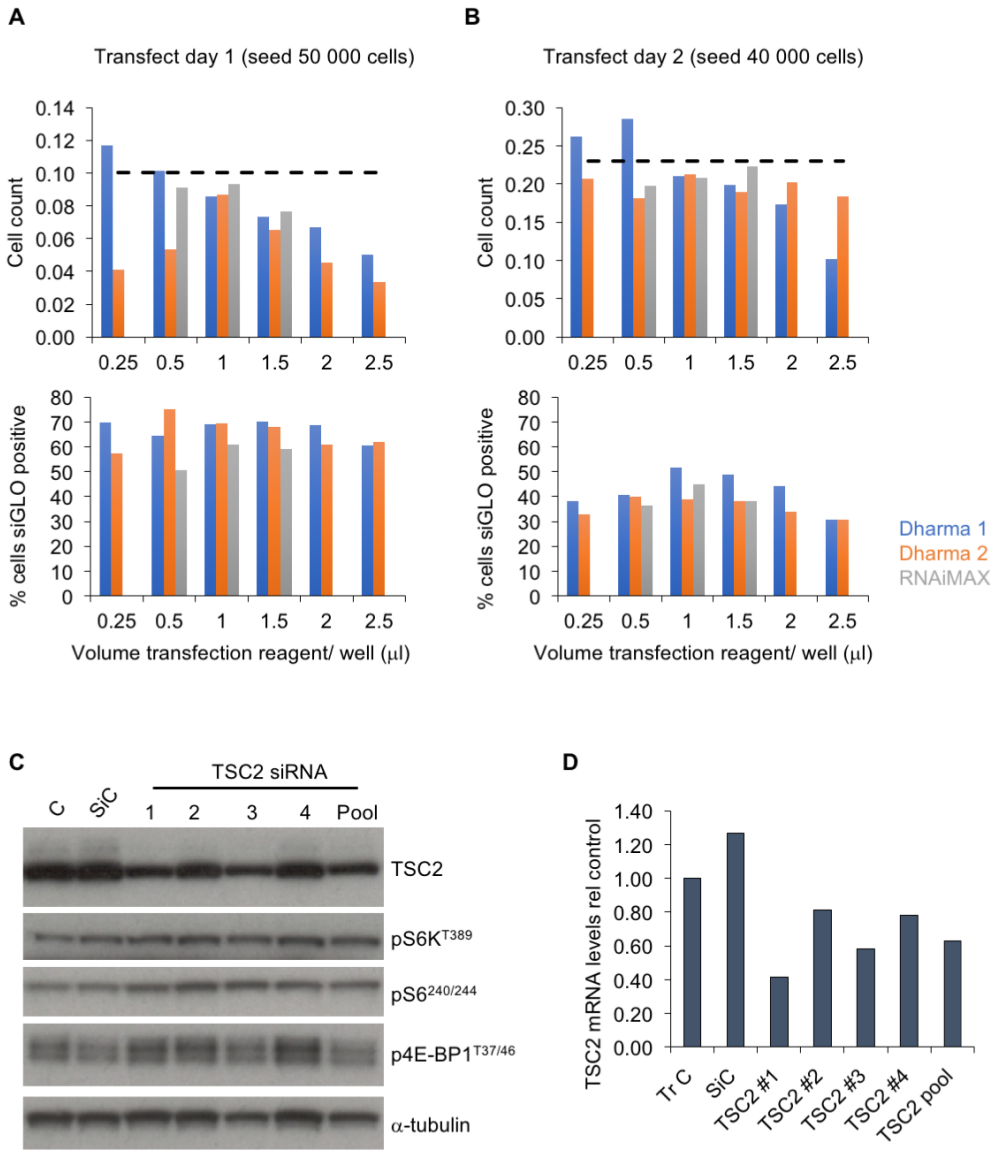


Figure 4.7: Optimisation of Tsc2 knockdown by siRNA.

A, B. Differentiating ESCs were transfected with siGLO fluorescent siRNA either 1 or 2 days after seeding, and cell viability and transfection efficiency was assessed to determine optimal transfection reagent, transfection time-point, and reagent concentration. Horizontal dotted line denotes cell number in untransfected control cells. C, D. Four siRNAs targeting Tsc2 were transfected into cells and TSC2 protein (C) and mRNA (D) levels were determined to select the siRNA which yielded greatest knockdown. n=1.

Transient knockdown of TSC2 partially rescues loser cell elimination

Knockdown of *Tsc2* at day 1 in competition resulted in a partial but statistically significant increase in the number of *Bmpr1a*$^{-/-}$ and 4n cells in competition at day 4 and, in *Bmpr1a*$^{-/-}$ cells,

at day 5 (Fig. 4.8A,B,D,E). This was accompanied by a partial rescue of pS6 levels in both lines during competition (Fig. 4.8C, F). These results suggest that the loss of mTOR activity in loser cells during competition is important for the elimination of defective cells.

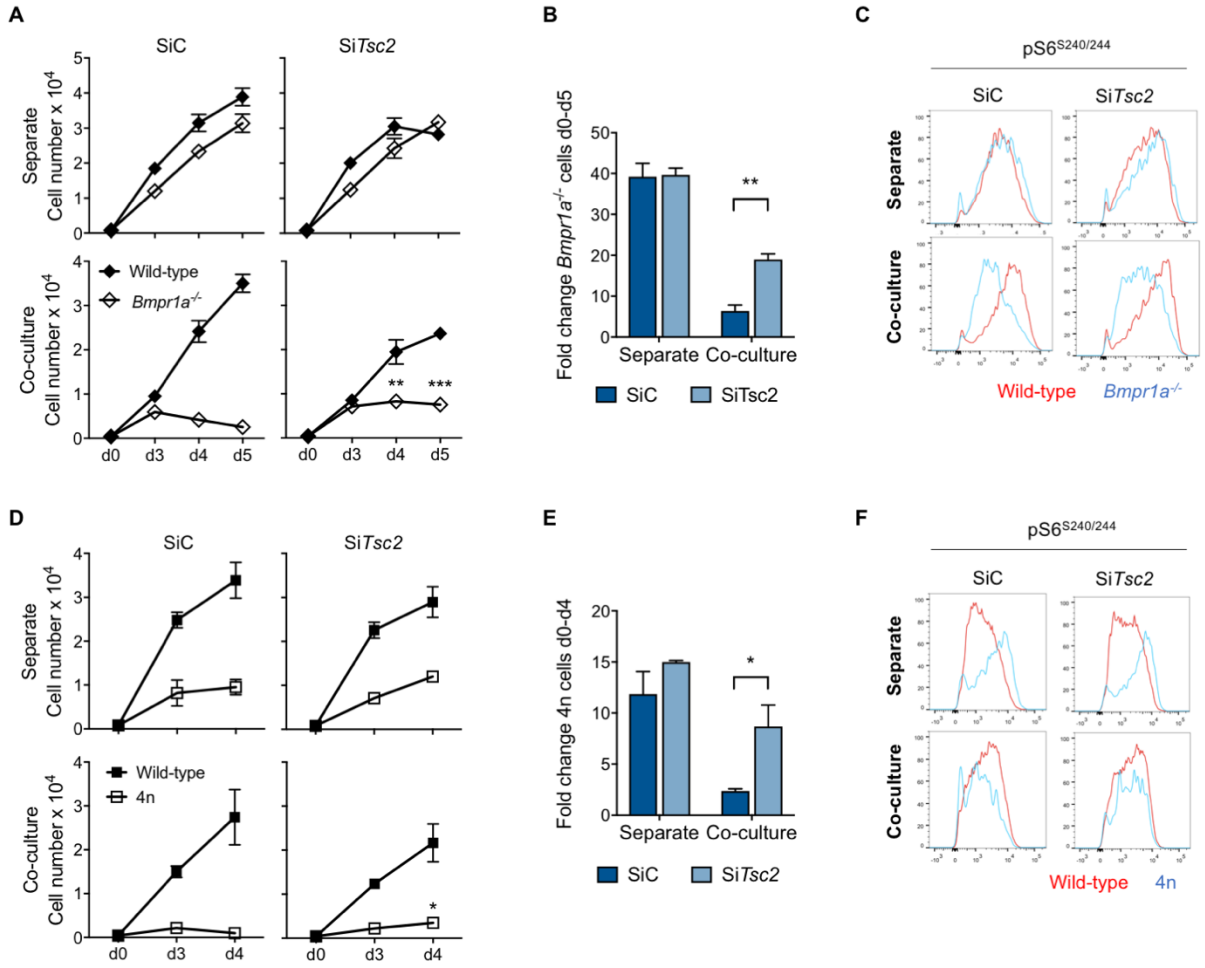


Figure 4.8: Knockdown of *Tsc2* by siRNA partially rescues loser cell elimination.

A. Growth curves of wild-type and *Bmpr1a*$^{-/-}$ cells in separate and co-culture treated with control (SiC) or *Tsc2* siRNAs. B. Fold change in *Bmpr1a*$^{-/-}$ cell number from d0-d4 when treated with control (SiC) or *Tsc2* siRNAs. C. pS6 staining in cells treated with control or *Tsc2* siRNA. D. Growth curves of wild-type and 4n cells in separate and co-culture treated with control (SiC) or *Tsc2* siRNAs. E. Fold change in 4n cell number from d0-d4 when treated with control (SiC) or *Tsc2* siRNAs. F. pS6 staining in wild-type and 4n cells treated with control or *Tsc2* siRNA.*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, two-tailed t-test (A,D comparing SiC loser cell number to Si*Tsc2*) or 2-way ANOVA followed by Sidak's post-hoc test (B,E). Error bars denote SEM. n=3 for all experiments.

Optimisation of shRNA protocol

Since siRNA knockdown is transient, it is likely that the efficacy of this approach is limited by the high rates of proliferation in differentiating ESCs. Therefore, we aimed to stably knockdown TSC2 by infecting cells with viruses expressing short-hairpin RNAs (shRNAs). First we infected cells with pGIPZ vectors expressing four different Tsc2-targetting shRNA oligos to test knockdown efficiency at the protein level by Western blot. We observed good knockdown of TSC2 using shRNA #2 when cells were maintained in pluripotent conditions in ESC media (Fig. 4.9A, left). However, 3 days of differentiation in N2B27 led to a reduction in knockdown efficiency, presumably as a result of silencing of the viral DNA (Fig. 4.9A, right). To better assess dynamics of the silencing of viral genes during differentiation, we infected cells with pGIPZ-GFP. Although in pluripotent conditions cells were selected so that >95% were GFP positive, by day 3 in N2B27 media only 30% of pGIPZ-GFP infected cells expressed GFP, and by day 4 this was reduced to 23% (Fig. 4.9B).

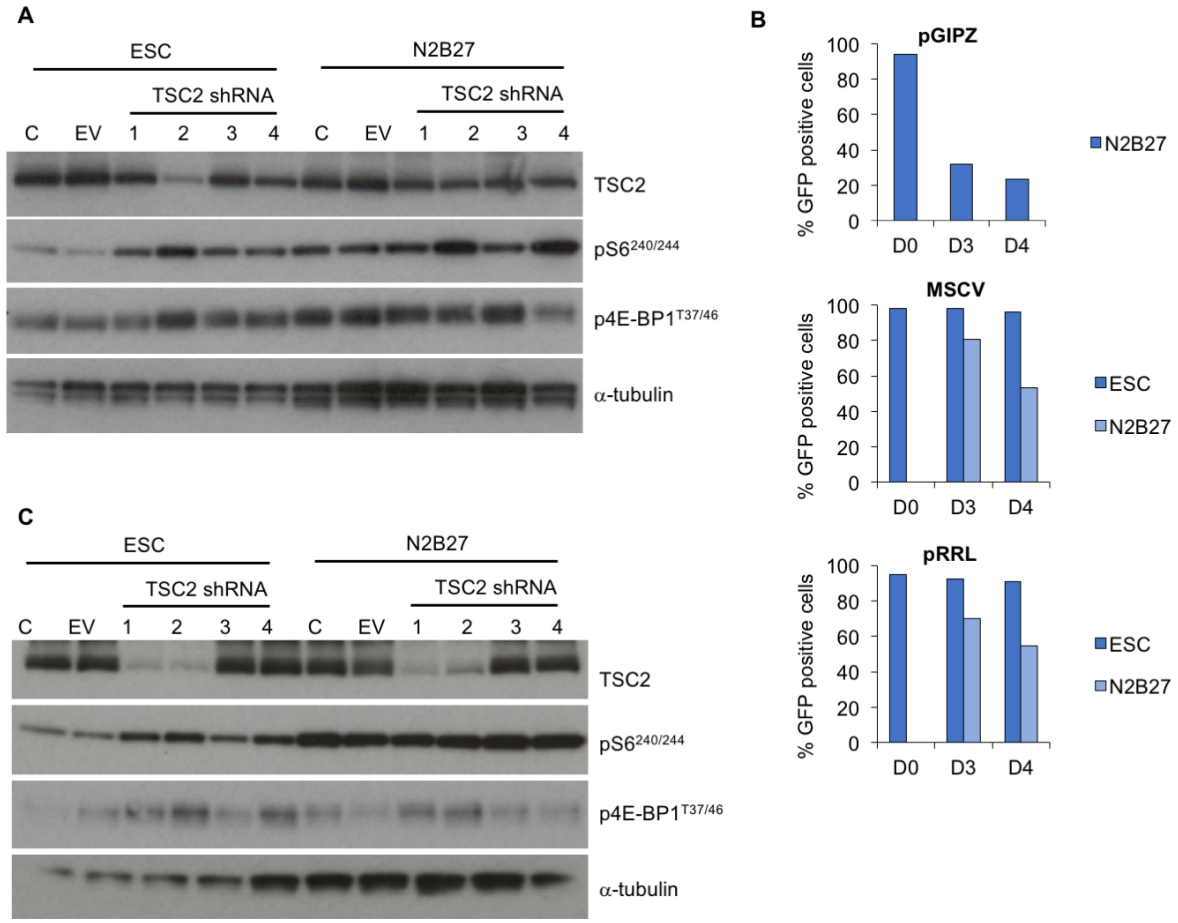


Figure 4.9: Optimisation of viral-mediated shRNA delivery.

A. pGIPZ vectors were used to deliver 4 different Tsc2-targetting shRNAs. After selection, cells were cultured in ESC or N2B27 media for 3 days and TSC2 protein levels were assessed by Western blot. B.

Analysis of GFP expression levels during differentiation in pGIPZ, MSCV and pRRL vectors. C. TSC2 levels in pluripotent (ESC) and differentiating (N2B27) ESCs following infection with MSCV vector carrying *Tsc2*-targetted shRNAs. n=1.

We therefore tested the expression of two other GFP-labelled viral vectors during differentiation: murine stem cell virus (MSCV) and pRRL, both of which contain an optimised miRE backbone to enhance shRNA processing and knockdown efficiency (Fellmann et al., 2013). Furthermore, the MSCV vector contains a modified promoter which should avoid silencing (Swindle et al., 2004). By day 3 in N2B27 media, 80% of cells infected with MSCV-GFP, and 60% of cells infected with pRRL-GFP, were GFP-positive (Fig. 4.9B). This indicated that silencing of viral DNA during differentiation was less great using these vectors. By day 4 in N2B27, 50% of cells infected with both vectors still expressed GFP, which was over double the proportion of cells infected with pGIPZ.

Since the MSCV vector exhibited less silencing of viral elements compared with pGIPZ, the *Tsc2*-targeted shRNA oligos were cloned into this vector for infection of *Bmpr1a*$^{-/-}$ cells. We observed good knockdown of TSC2 using the MSCV vector in pluripotency conditions with oligo #1 and #2, and knockdown was sustained during differentiation (Fig. 4.9C).

Stable knockdown of Tsc2 rescues Bmpr1a$^{-/-}$ *cell elimination*

Knockdown of Tsc2 in *Bmpr1a*$^{-/-}$ cells using shRNAs resulted in a rescue of *Bmpr1a*$^{-/-}$ out-competition by wild-type cells (Fig. 4.10A,B) and a rescue of pS6 levels (Fig. 4.10C, D). This result indicates that loss of mTOR activity in *Bmpr1a*$^{-/-}$ cells in a competitive environment is required for their elimination. As it has been reported that mouse embryonic stem cells require TSC2 to exit pluripotency (Betschinger et al., 2013), a possible interpretation of these results is that reduction of TSC2 levels has prevented cell differentiation, which has indirectly blocked cell competition. However, we analysed several markers of differentiation after three days in N2B27 at the mRNA level and found them to be similar in *Tsc2*-knockdown cells compared with empty vector-infected cells (Fig. 4.10E), and significantly lower than the pluripotent control. We also analysed expression of cell-surface early differentiation marker CD31 by flow cytometry and found it to be reduced in all lines compared to an undifferentiated control (Fig. 4.10F). We conclude that the rescue of *Bmpr1a*$^{-/-}$ outcompetition by *Tsc2* knockdown is independent of differentiation.

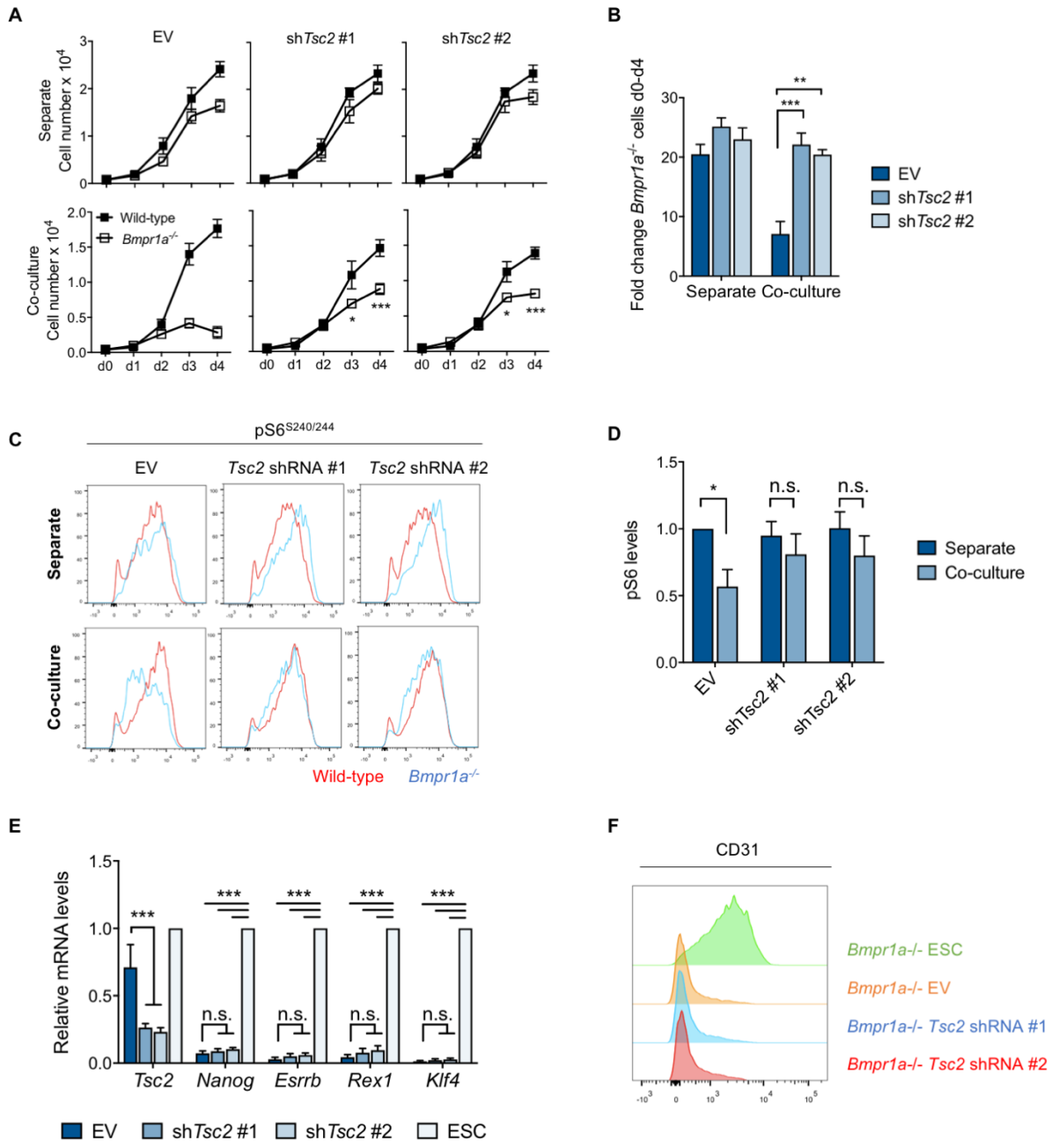


Figure 4.10: Knockdown of Tsc2 rescues elimination of *Bmpr1a*$^{-/-}$ cells.

A. Growth curves of wild-type and *Bmpr1a*$^{-/-}$ cells in separate and co-culture, where *Bmpr1a*$^{-/-}$ cells are infected with MSCV containing non-targeting empty vector (EV) or *Tsc2*-targeting shRNAs. B. Fold change in *Bmpr1a*$^{-/-}$ cell number in separate and co-culture when infected with EV or shRNAs targeting Tsc2. C, D. pS6 staining in *Bmpr1a*$^{-/-}$ infected with EV or *Tsc2*-targeting shRNAs cultured with and without wild-type cells. E,F. Analysis of pluripotency markers in *Bmpr1a*$^{-/-}$ cells infected with EV or Tsc2-targetting shRNAs, relative to ESC pluripotent control, assessed by qPCR (E) or flow cytometry (F). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, two-tailed t-test (C comparing EV cell number to sh*Tsc2*) or 2-way ANOVA followed by Sidak's (F) or Tukey's (D,G) post-hoc test. Error bars denote SEM. n=3.

Using CRISPR to knockout Tsc2 in 4n cells

To assess whether mTOR is also functionally important in our tetraploid model of cell competition, we attempted to hyper-activate the pathway in these cells. We were not able to induce good knockdown of TSC2 using shRNAs in these cells (Fig. 4.11A), and so used CRISPR/Cas9 to disrupt the gene. As the tetraploid cells were already puromycin-resistant due to the GFP label, we first introduced the Cas9 enzyme into cells using a lenti-Cas9-blasticidin viral vector (Fig. 4.11B, left). Following infection, cells were seeded at clonal density, selected using blasticidin, and 13 clones were screened by Western blot for expression of FLAG, which was conjugated to Cas9 (Fig. 4.11B, right). All 13 clones expressed FLAG. We picked one to grow up and infect with *Tsc2*-targeting gRNAs.

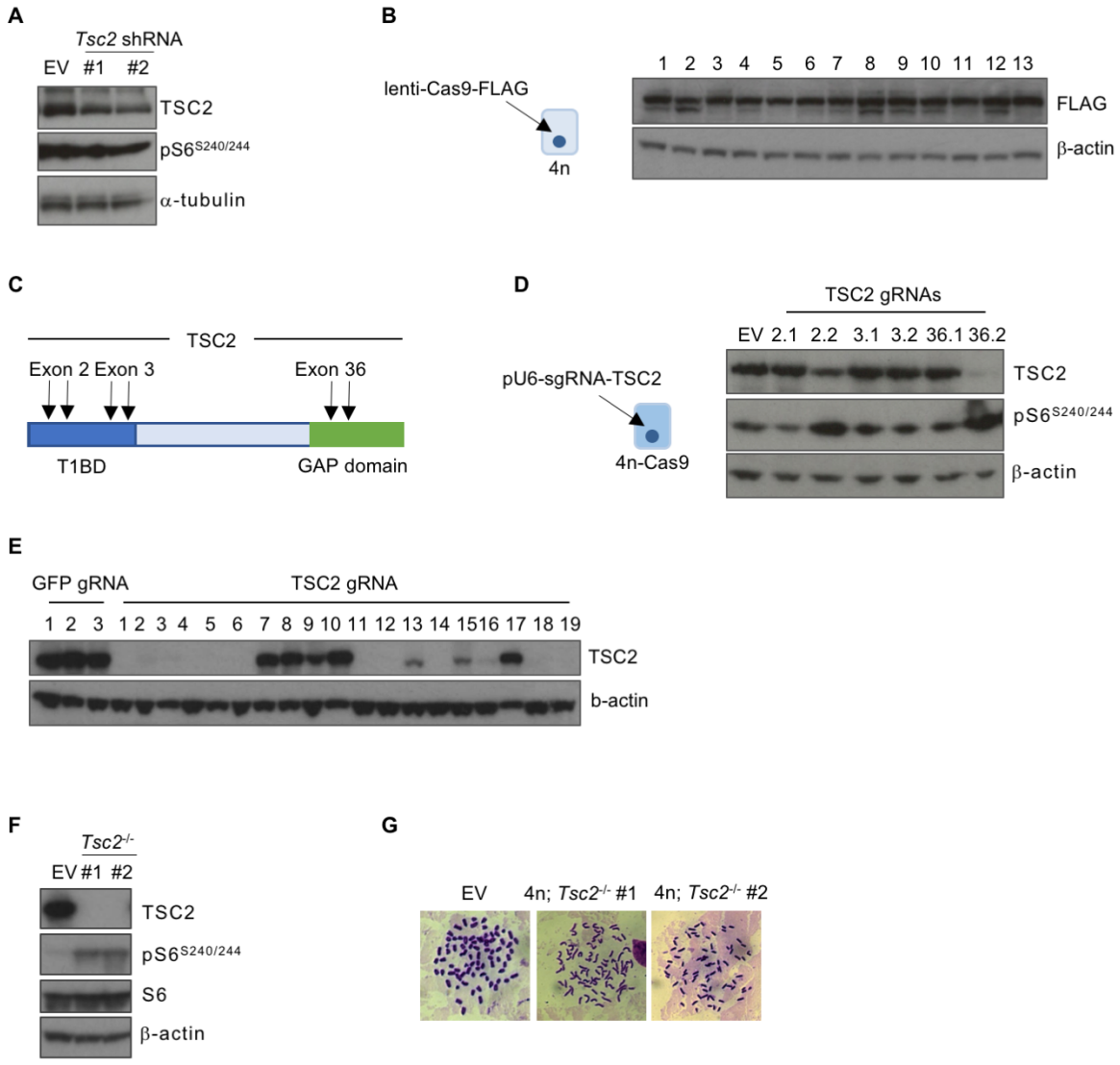


Figure 4.11: Generation of *Tsc2*$^{-/-}$ 4n cells.

A. Knockdown of Tsc2 by shRNA in 4n cells. B. 4n cells were infected with lenti-Cas9 vector expressing FLAG-tagged Cas9 enzyme and 13 clones generated from single cells were screened for

expression of FLAG. C. Cartoon depicting gRNA target sites on *Tsc2* gene. D. 4n-Cas9 cells from (B) were infected with viruses carrying gRNAs targeting 6 different target sites on Tsc2 (as in C). TSC2 protein levels in pools of selected cells were analysed by Western blot. E. 4n-Cas9 cells were infected with viruses expressing gRNAs targeting either GFP as a control or exon 36 of *Tsc2*. Cells were seeded at single cell density and 19 clones were screened for loss of TSC2 protein. F. Two 4n *Tsc2*$^{-/-}$ clones were selected and pS6 levels were analysed. G. 4n-*Tsc2*$^{-/-}$ clones maintained tetraploidy.

Initially, 6 gRNAs targeting *Tsc2* were designed: 4 targeted exons number 2 and 3 in the TSC1-binding domain, and 2 targeted exon number 36 in the functional GAP domain (Fig. 4.11C). Disruption of these domains have previously been reported to result in loss of the protein (Fu and Ess, 2013; Onda et al., 1999). To assess which gRNA was most effective at reducing TSC2 protein levels, we infected cells with the gRNAs, selected stable integrants using hygromycin, and analysed TSC2 protein expression in the pool of cells by Western blot (Fig. 4.11D). This revealed that the gRNA most effective at reducing Tsc2 protein levels was that targeting exon 36 (36.2). Therefore, we infected 4n-Cas9 cells with a lentiviral vector carrying this gRNA, seeded cells at clonal density, and picked colonies to screen for loss of TSC2. Out of 19 colonies picked, 12 had undetectable levels of TSC2 (Fig. 4.11E). We picked two of these clones for expansion for further competition analysis (Fig. 4.11F), after confirming that they were still tetraploid by karyotyping (Fig. 4.11G).

Knockout of Tsc2 rescues 4n cell elimination

We observed a complete rescue of tetraploid elimination in clones lacking TSC2 in competition (Fig. 4.12A,B), and a rescue of pS6 levels (Fig. 4.12C,D). To our surprise, it was observed that tetraploid clones grew better than wild-type cells in co-culture, and that wild-type cells arrested their growth from day 3 onwards (Fig. 4.12A,B). Complete knockout of *Tsc2* resulted in a delay in differentiation: at day 3 in N2B27 we observed increased CD31 staining compared with tetraploid GFP-targeted controls (Fig. 4.12F). However, by day 4 in N2B27, mRNA pluripotency markers were dramatically reduced compared to a pluripotent control, and this decrease was similar to control tetraploid cells (Fig. 4.12E). We therefore find that tetraploid cell elimination can also be rescued by preventing loss of mTOR pathway activation, and that this is independent of cell differentiation.

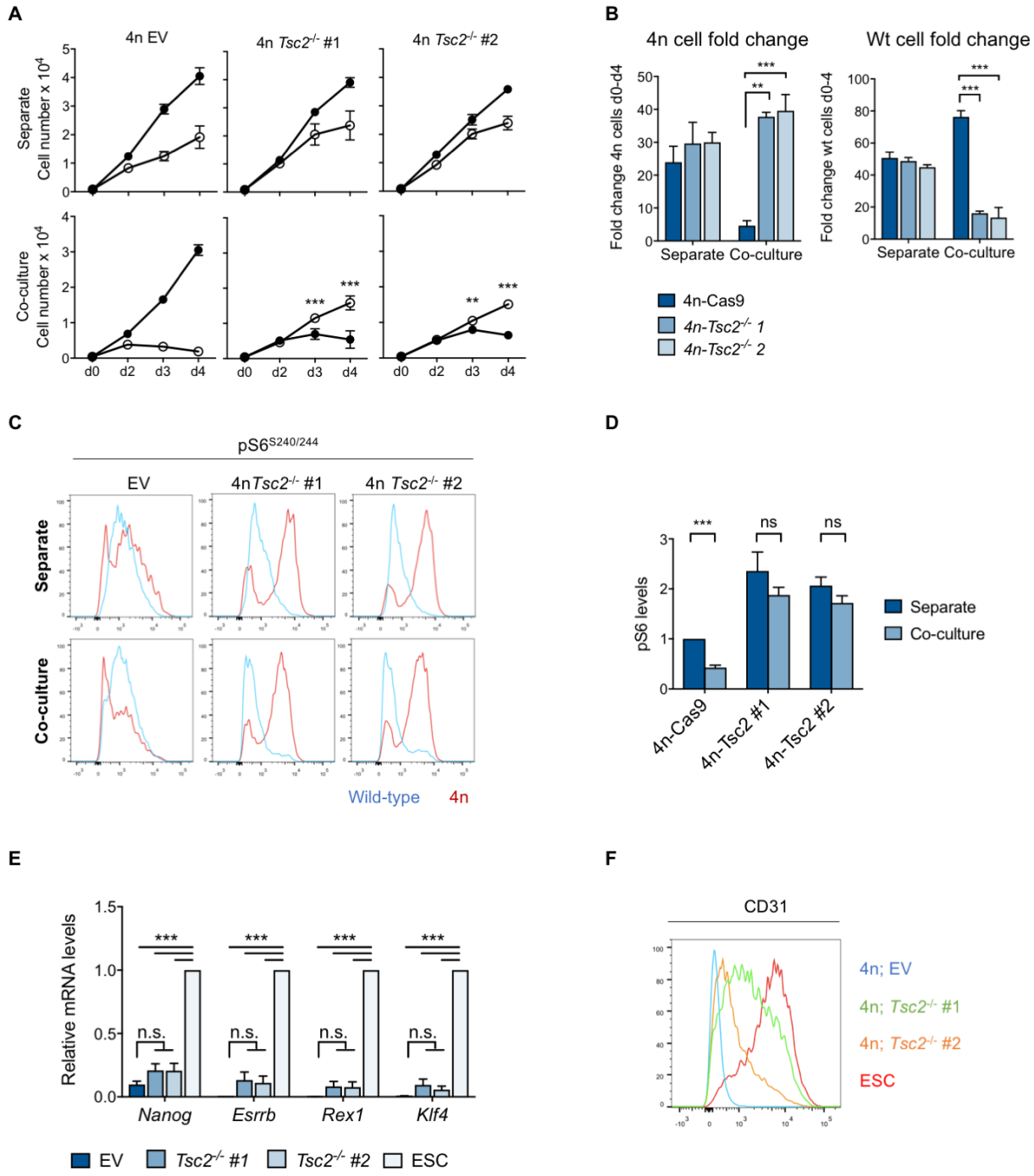


Figure 4.12: Knockout of Tsc2 rescues elimination of 4n cells.

A. Growth curves of wild-type cells and control and *Tsc2*$^{-/-}$ 4n cells in separate and co-culture. B. Fold change in control and *Tsc2*$^{-/-}$ 4n cells relative to seeding number. C. pS6 staining in control and *Tsc2*$^{-/-}$ 4n cells and quantification (D). E,F. Analysis of pluripotency markers in control and *Tsc2*$^{-/-}$ 4n cells, relative to ESC pluripotent control, assessed by qPCR (E) and flow cytometry (F). **, $p<0.01$; ***, $p<0.001$, two-tailed t-test (A comparing EV cell number to 4n-*Tsc2*$^{-/-}$), 1-way ANOVA followed by Tukey's post-hoc test (D) or 2-way ANOVA followed by Tukey's (B,E) post-hoc test. Error bars denote SEM.

4.2.4. Downstream of mTOR

Overexpression of S6 kinase 1

We next asked if constitutive activation of an mTOR target, S6 kinase, was sufficient for the rescue of defective cell elimination. In order to perform this experiment, we used a mutant form of S6 kinase which lacks the F5A inhibitory C-terminus and contains a T389A point mutation to mimic constitutive phosphorylation (hereafter referred to as 'CA-S6K'; Schalm and Blenis, 2002). This was cloned into a pyCAG vector and introduced into cells by lipofection. Initially, we transiently transfected wild-type cells to test whether expression of the protein was sufficient to override rapamycin-induced inhibition of S6 phosphorylation. We found that under normal conditions, S6 phosphorylation in CA-S6K-transfected cells was increased compared to untransfected controls (Fig. 4.13A). Furthermore, while rapamycin treatment abolished S6 phosphorylation in normal cells, CA-S6K-transfected cells were partially resistant to treatment. This indicated that transient transfection of cells with CA-S6K induced pS6 hyper-phosphorylation and reduced rapamycin sensitivity.

Next, we selected clones of tetraploid cells with stable integration of the CA-S6K plasmid. Four clones expressing different levels of the protein were selected and levels of pS6 and pS6K were assessed by Western blot. We observed a variation in the levels of S6K expression, as indicated by HA, and of pS6/ pS6K (Fig. 4.13B). However, none of the clones were insensitive to out-competition by wild-type cells (example shown in Fig. 4.13C). Furthermore, we did not observe a rescue of pS6 levels in competing tetraploid cells (Fig. 4.13D). To assess whether stable expression of S6 kinase rendered cells insensitive to rapamycin treatment, we treated 3 clones with rapamycin. In contrast to the transient transfection, all S6K-expressing clones lost pS6 expression to similar levels as control cells (Fig. 4.13E). Together these results suggest that when stably expressed, constitutively active S6 kinase cannot override mTOR inhibition. This could be through the activation of compensatory mechanisms.

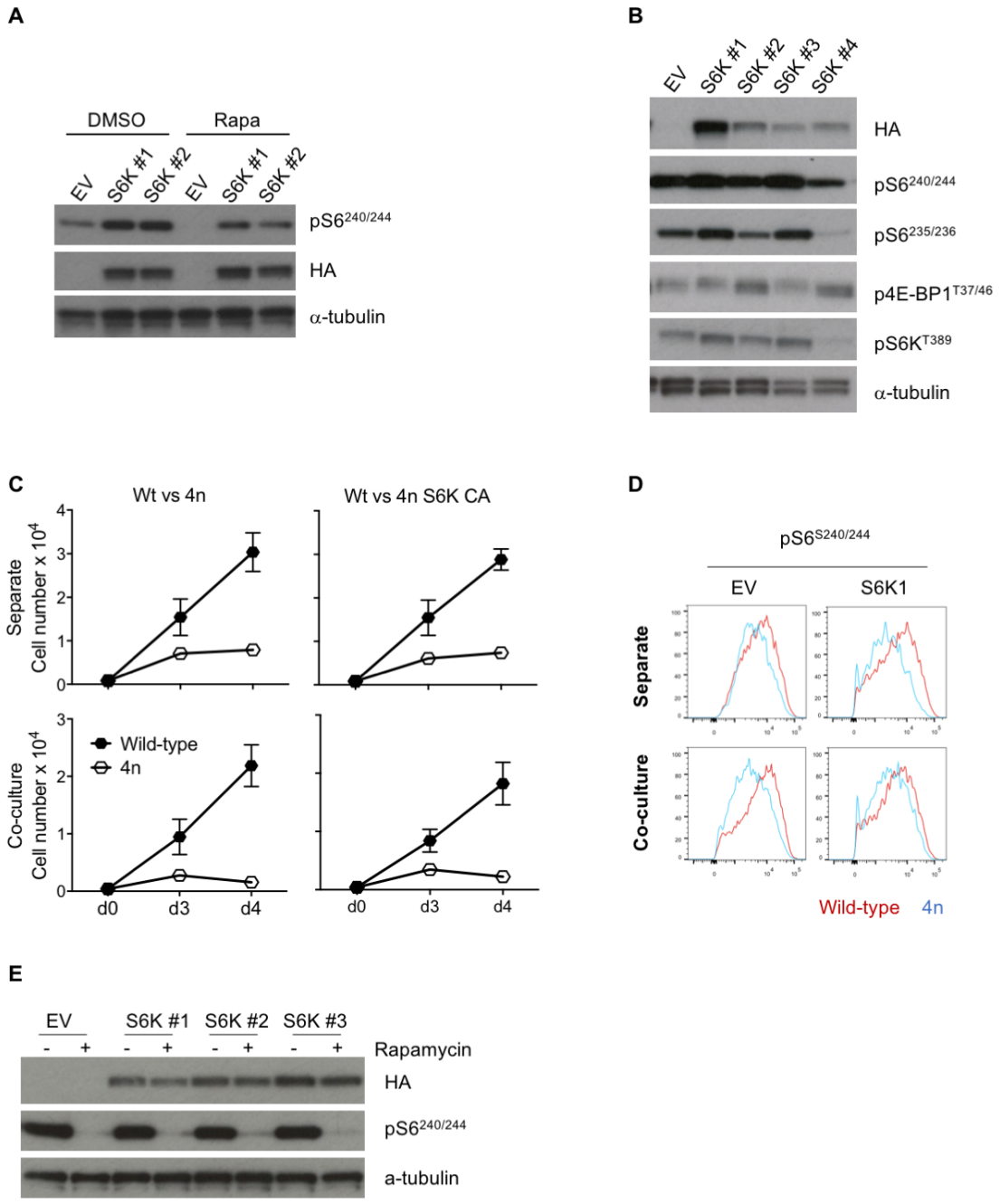


Figure 4.13: Overexpression of constitutively active S6 kinase does not rescue competition.

A. Rapamycin treatment (1μM, 2hr) in wild-type cells transiently transfected with constitutively active S6 kinase. B. 4n cells were transfected with HA-tagged constitutively active S6 kinase and selected for stable integrants. 4 clones were screened for expression of HA-tag. C. Growth curves of wild-type and control/ S6K-expressing 4n cells in separate and co-culture. D. pS6 levels of wild-type and control/ S6K-expressing 4n cells in separate and co-culture. E. Rapamycin treatment (1uM, 2hr) in wild-type cells stably transfected with constitutively active S6 kinase. Error bars denote SEM.

Inhibition of autophagy

Autophagy is another significant downstream component of the mTOR pathway and this pathway was upregulated in the IPA analysis (Fig. 4.1B) in *Bmpr1a*$^{-/-}$ cells in co-culture compared with wild-type cells. We therefore aimed to assess whether loser cell elimination relied on high levels of autophagy induced in response to mTOR inhibition. We knocked down autophagy regulator and mTOR target Ulk1 in *Bmpr1a*$^{-/-}$ cells and assessed cell competition dynamics. Although two Ulk1-targetting shRNAs resulted in mRNA knockdown of about 70% (Fig. 4.14A), no rescue of *Bmpr1a*$^{-/-}$ elimination was observed (4.14B). These results suggest that an induction of autophagy following mTOR pathway repression in loser cells is not required for loser cell elimination. In order to confirm these results we would need to verify that autophagy flux has been reduced; this could be done through analysis of LC3 or p62 levels by Western blot following knockdown of Ulk1.

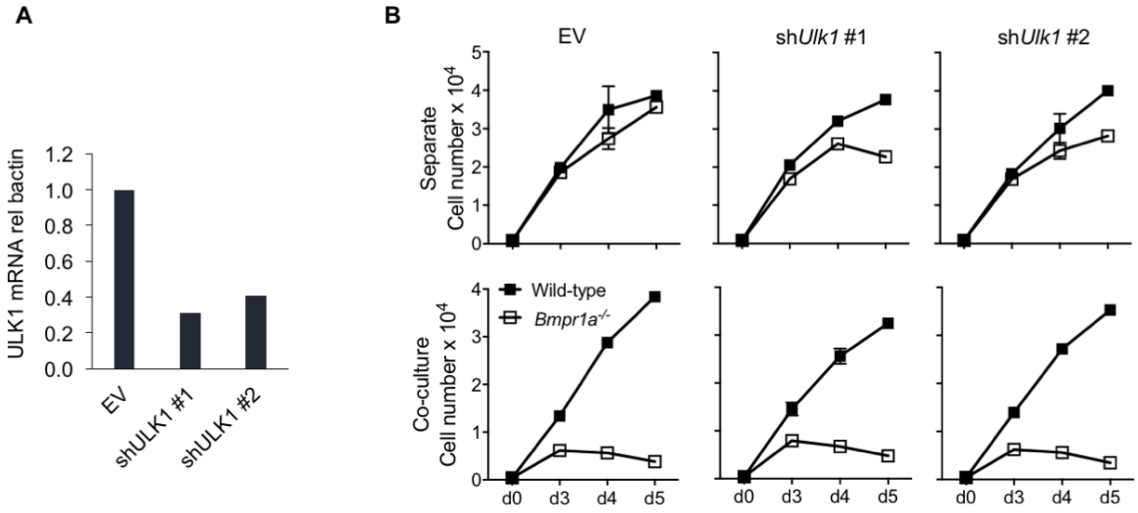


Figure 4.14: Repression of autophagy does not affect competition dynamics.

A. mRNA levels of *Ulk1* in *Bmpr1a*$^{-/-}$ cells following infection with MSCV containing *Ulk1*-targeting shRNAs. n=1. B. Growth curves of wild-type and *Bmrp1a*$^{-/-}$ in separate and co-culture in empty vector (EV) *Bmpr1a*$^{-/-}$ and *Ulk1*-knockdown *Bmpr1a*$^{-/-}$ cells. Error bars denote SEM. n=2

Phosphorylation of S6K or 4EBP1

Since rapamycin treatment triggered cell apoptosis (Fig. 4.6), we asked if repression of either S6 kinase or 4E-BP1 phosphorylation downstream of mTOR is sufficient to induce cell death. To assess the importance of S6 kinase activity in cell viability, we treated cells with S6 kinase inhibitor PF-4708671. This inhibitor has been described to have high specificity towards S6 kinase (Pearce et al., 2010). Consistent with this, we observed a dose-dependent reduction in

pS6 in both pluripotent and differentiation conditions (Fig. 4.15A). However, unlike inhibition of the mTOR pathway using rapamycin, we did not observe cell death in response to inhibition of S6 kinase activity. In order to assess the importance of 4E-BP1 phosphorylation for cell viability, we infected cells with viruses expressing a dominant negative 4EBP1 transgene which is unable to undergo phosphorylation, and therefore mimics conditions in which mTORC1 is repressed. Cells expressing this gene did not exhibit increased levels of cell death (Fig. 4.15B). Furthermore, rapamycin treatment raised cleaved caspase-3 levels to a similar degree in empty vector (EV) control cells and cells expressing dominant negative 4EBP1. We conclude that repression of neither S6K nor 4E-BP1 phosphorylation downstream of mTOR is sufficient to drive elimination of cells, and that another mTOR target or a combination of targets is required for the induction of apoptosis.

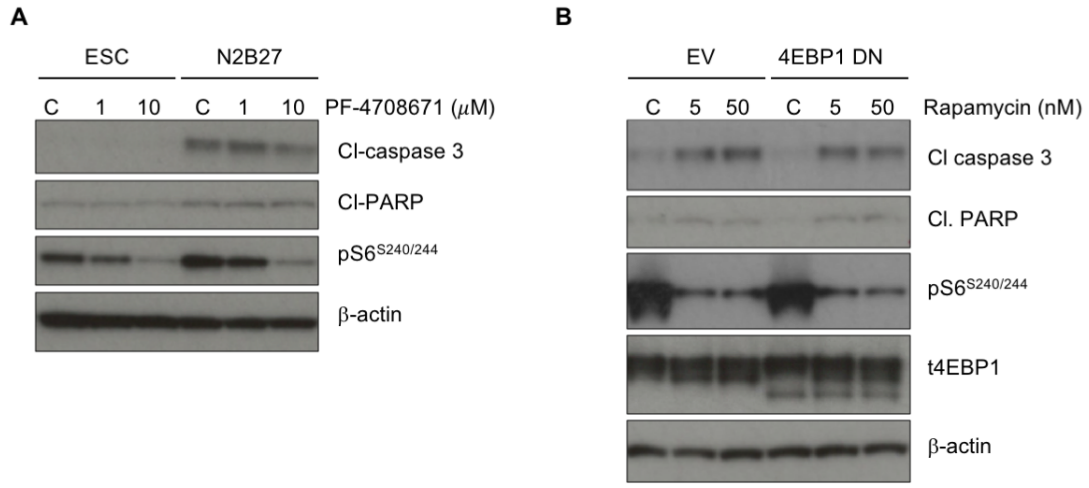


Figure 4.15: Inhibition of either S6 kinase or 4E-BP1 is not sufficient to induce cell apoptosis.
A. Cells were treated with S6 kinase inhibitor PF-4708671 for 6 hours and levels of cell death markers cleaved caspase-3 and cleaved PARP were assessed by Western blot. n=2. B. Cells were infected with viruses expressing a dominant negative 4E-BP1 and cell death markers cleaved caspase-3 and cleaved PARP were assessed by Western blot. n=2.

4.2.5. Differences in mTOR pathway activation are sufficient to induce cell competition

Our results have revealed that loss of mTOR activation is both sufficient and necessary for loser cell elimination during cell competition, so we asked whether competition could be triggered by differential levels of mTOR activation alone. We used CRISPR to knockout *Tsc2* in wild-type cells (Fig. 4.16A), and cultured these *Tsc2*$^{-/-}$ cells with wild-type ESCs. We found that two independent clones of cells lacking TSC2 grew similar to wild-type cells in separate culture, but induced competition (the elimination of wild-type cells) in co-culture (Fig. 4.16B-

D). The fold-change of wild-type cells over 5 days in co-culture was significantly reduced compared to Cas9 controls (Fig. 4.16B,C). Furthermore, the proportion of cleaved caspase-3-positive wild-type cells doubled in the presence of *Tsc2*$^{-/-}$ cells (Fig. 4.16D). Wild-type cells did not lose pS6 staining when cultured with *Tsc2*$^{-/-}$ cells, indicating that their elimination is not mediated through non-autonomous inhibition of the mTOR pathway (Fig. 4.16E). Finally, levels of pluripotency marker CD31 were similar in wild-type and *Tsc2*$^{-/-}$ cells at d3 suggesting that the *Tsc2*-mediated super-competition is not due to differences in cell differentiation (Fig. 4.16F).

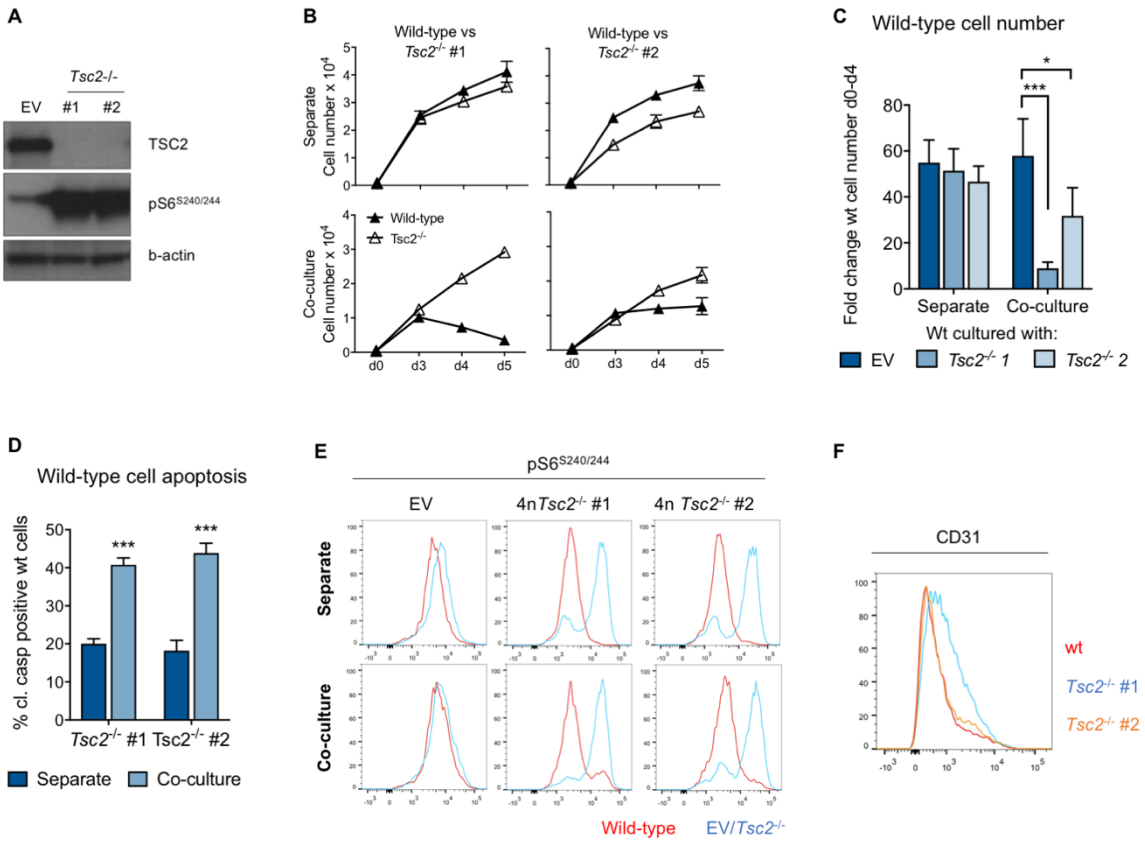


Figure 4.16: Differences in mTOR pathway activity are sufficient to drive competition.

A. *Tsc2*$^{-/-}$ lines were generated using CRISPR and two clones of cells were selected and expanded. B. Wild-type and *Tsc2*$^{-/-}$ cells were cultured separately and together for 5 days in N2B27 and cell proportions and counts were assessed daily from d3-d5. C. Fold change in wild-type cell number from seeding number to number at end of assay when cultured alone or with Cas9 control or *Tsc2*$^{-/-}$ cells. D. Cl. caspase-3 levels in wild-type cells when cultured alone or with *Tsc2*$^{-/-}$ cells were assessed by flow cytometry. E. pS6 levels in wild-type and EV/*Tsc2*$^{-/-}$ cells when cultured alone and together were assessed by flow cytometry at day 4. F. Levels of pluripotency marker CD31 in wild-type and *Tsc2*$^{-/-}$

cells were assessed by flow cytometry at day 3. *, $p<0.05$; ***, $p<0.001$, 2-way ANOVA followed by Sidak's (C,D) post-hoc test. Error bars denote SEM. n=3.

4.2.6. mTOR activity is important in cell competition *in vivo*

Our *in vitro* system of cell competition is designed to model embryonic differentiation. Day 3 of competition (when we observe loss of mTOR pathway activation in loser cells) is roughly equivalent to the E5.5 embryo and day 4 to the E6.5 embryo, when a wave of cell death occurs during early embryogenesis. To identify whether cell competition *in vivo* results in loss of mTOR activation in loser cells, we stained E5.5 wild-type/ 4n chimeric embryos for $pS6^{S240/244}$. The loser tetraploid cells showed reduced, or even absent, staining of $pS6^{S240/244}$, confirming that the differential activation of the mTOR pathway observed *in vitro* also occurs *in vivo* (Fig. 4.17A).

Next, we generated chimeras of 4n-*Tsc2*$^{-/-}$ cells to identify if hyper-activation of the mTOR pathway rescued cell elimination. As expected, $pS6^{S240/244}$ levels were rescued in these chimeras (Fig. 4.17B). At E6.5, 4n cells undergo apoptosis and stain positively for cleaved caspase-3 (Fig. 4.17C,D; Sancho et al., 2013). However, 4n-*Tsc2*$^{-/-}$ cells had significantly reduced cleaved caspase staining compared to 4n cells (Fig. 4.17C,D). Next we aimed to identify if hyper-activation of the mTOR pathway allowed persistence of tetraploid cells to later stages in development. As *Tsc2*$^{-/-}$ mutations are embryonic lethal from E9.5 (Onda et al., 1999), we assessed contribution of tetraploid cells at E9.0. We observed high contribution of GFP-labelled 4n; *Tsc2*$^{-/-}$ cells to chimeras at E9.0 (Fig. 4.17E). However, these chimeras exhibited significant growth delay and resembled E8-8.5 embryos. This could be either because of the negative effects of constitutive mTOR activation on development or because of the negative consequences that the persistence of tetraploid cells has on embryogenesis.

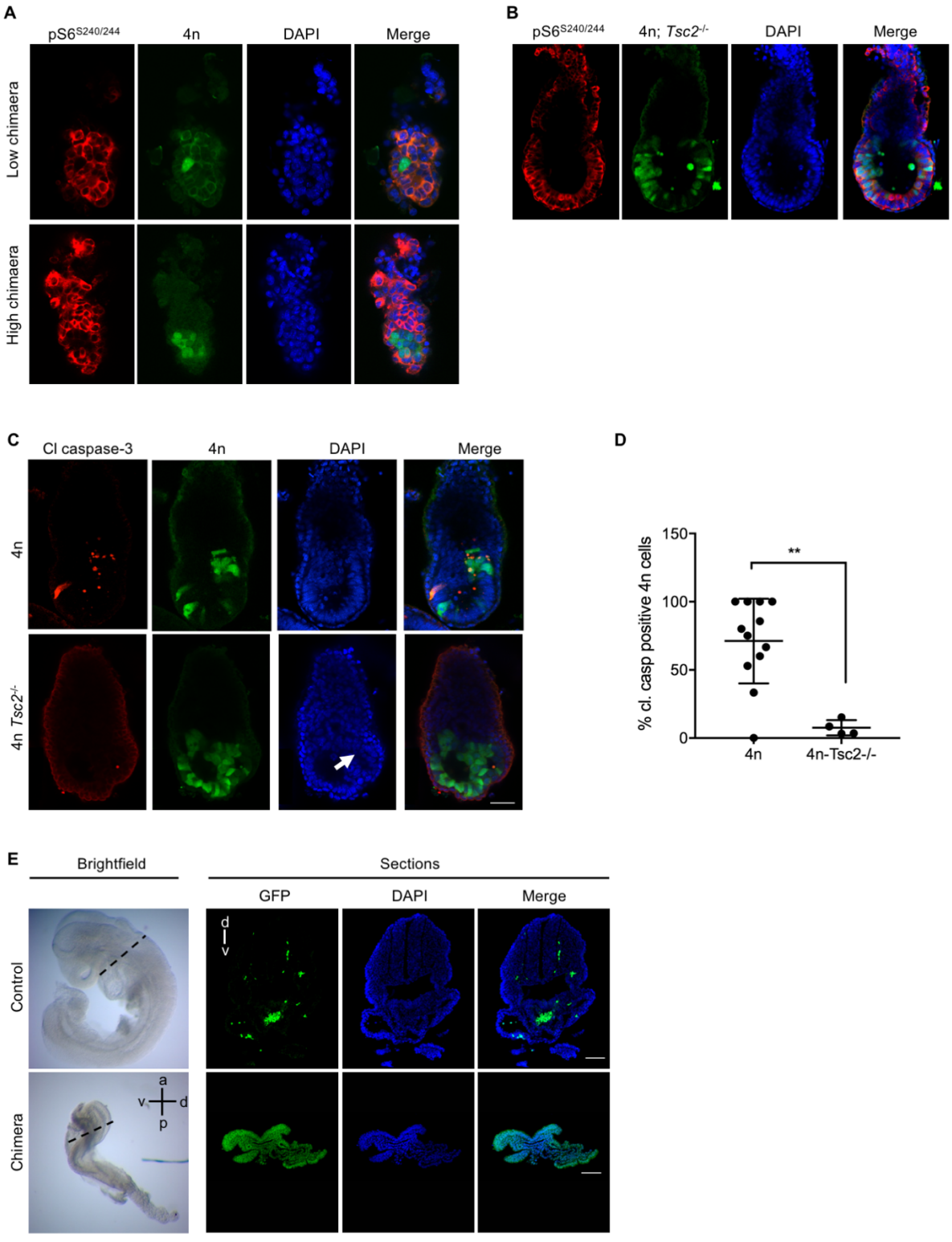


Figure 4.17: *Tsc2*$^{-/-}$ 4n cells are able to contribute to chimeric embryos *in vivo*.

A. 4n cells were injected into wild-type blastocysts, dissected at E5.5, and stained for pS6$^{S240/244}$. n=5. B. 4n; *Tsc2*$^{-/-}$ cells were injected into wild-type blastocysts, dissected at E6.5 and stained for pS6$^{S240/244}$. n=3. C. 4n (n=12) or wild-type/4n; *Tsc2*$^{-/-}$ (n=4) cells were injected into wild-type blastocysts, dissected at E6.5 and stained for cleaved caspase-3 and quantified (D). E. 4n; *Tsc2*$^{-/-}$ cells (GFP) were injected

into wild-type blastocysts, dissected at E9.0 and morphology and 4n;*Tsc2*$^{-/-}$ cell contribution was assessed. n=3. **, $p<0.01$, two tailed t-test. Error bars denote standard deviation. Chimeras were generated in collaboration with Sara Pozzi and Juan-Pedro Martinez Barbera (UCL) and were dissected, stained and imaged by Aida di Gregorio (Imperial College London).

4.3. Discussion

4.3.1. mTOR as a general sensor of cell competition

In summary, we have found that in response to a competitive environment, loser cells downregulate mTOR pathway activity. This is necessary for cell competition, as hyperactivation of the pathway rescues cell elimination, but is also sufficient for cell elimination during competition to occur, as repression of the mTOR pathway in differentiating cells triggers apoptosis. Furthermore, differences in mTOR signalling are sufficient to trigger out-competition of even wild-type cells. *Bmpr1a*$^{-/-}$ cells and tetraploid cells are both eliminated in competition with wild-type cells, although their defects are very different. Since mTOR is reduced in two different loser lines during cell competition, our results suggest that reduced mTOR levels could be a general sensor of competition in response to a variety of different defects which reduce cell competitiveness.

4.3.2. How does mTOR sense competition?

A question stemming from these data is: how does the mTOR pathway sense these different cell defects specifically in co-culture? Here I will outline three possible models which detail mechanisms that could lead to mTOR repression in defective cells: that cells are competing for shared nutrients; that stress is acting on loser cells in competition; or that a fitness-sensing event leads to mTOR repression (Fig. 4.18).

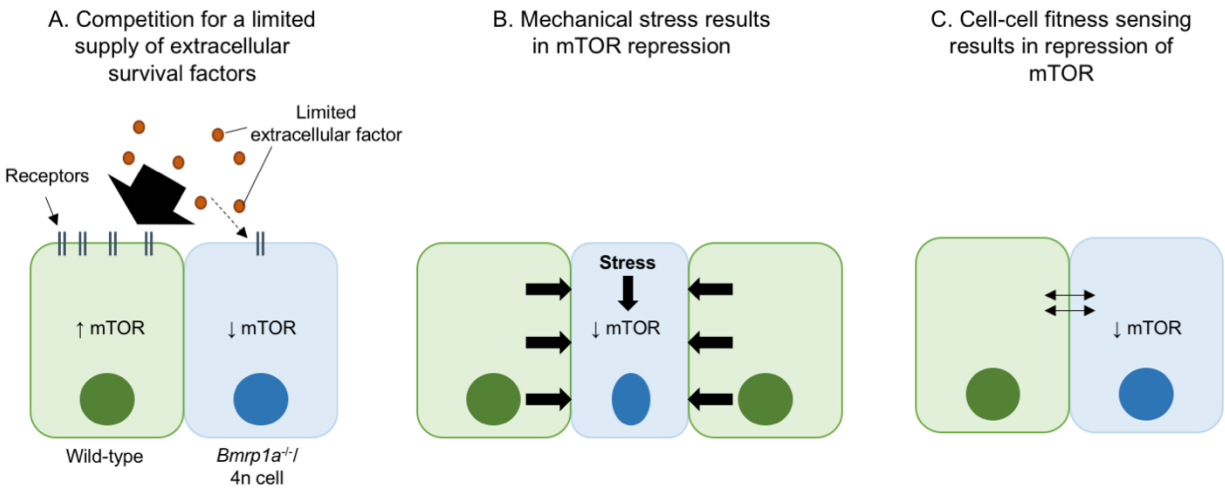


Figure 4.18: Possible models which could account for reduced activation of the mTOR pathway in loser cells specifically in co-culture.

A. Passive mechanisms could be responsible, whereby cells compete for a limited supply of extracellular factors necessary for survival. A competitive disadvantage could be conferred by fewer

cell surface receptors or reduced signal internalisation/ transduction. B. Stress undergone specifically in a competitive scenario, such as that triggered by mechanical forces, could repress mTOR signalling in defective cells. C. Cell-cell fitness sensing could result in repression of the mTOR pathway, either from a signal released from the winner cell or through loser autonomous recognition of reduced fitness.

One possibility is that passive mechanisms are at play. For example, as the mTOR pathway is activated in response to growth factors and nutrients, it is possible that loser cells compete with wild-type cells for a limited pool of extracellular factors (Fig. 4.18A). Elimination of loser cells could occur if these cells have a reduced ability to take up or transduce factors. In the absence of cell competition, the loser cells could be able to uptake nutrients and transduce the signals through mTOR, albeit at a slow rate. However, upon confrontation with wild-type cells, the loser cells could have a competitive disadvantage in taking up factors, resulting in loss of mTOR activity and death. This mechanism would bear similarities with those described in other models of competition, such as competition for IL-7 between T-cells (Martins et al., 2014), and for glucose between tumours and T-cells (Chang et al., 2015). The latter example also results in a reduction in mTOR activity in the outcompeted cell type (Chang et al., 2015). Similarly, mTOR repression could be a result of competition for oxygen resulting in hypoxia, which is known to negatively regulate the pathway (Brugarolas et al., 2004; DeYoung et al., 2008).

The mTOR pathway is also repressed by a wide range of proteins which are activated in response to stress. Among these is p53 (Feng et al., 2005), which has a role in sensing mechanical stress in MDCK cell competition (Wagstaff et al., 2016). Therefore, alternatively, it is possible that loss of mTOR activation occurs as a result of p53 activation in response to mechanical or other stress (Fig. 4.18B).

Another possibility is that mTOR repression occurs as a result of active fitness sensing mechanisms (Fig. 4.18C). For example, following recognition of a neighbouring defective cell, a wild-type cell could send out a signal which results in mTOR repression. Alternatively, following recognition of a neighbouring more fit cell, the defective cell could initiate a signalling cascade which results in mTOR repression. Similar mechanisms have been demonstrated in *Drosophila* epithelial competition, where for example molecular 'recognition' of *Scribble*$^{-/-}$ cells by PTP10D-Sas interaction induces a signalling cascade which results in JNK activation and cell death (Yamamoto et al., 2017). Deciphering between possible

upstream instigators of mTOR repression in competition will be the subject of the next thesis chapter.

4.3.3. Sensitivity of differentiating cells to mTOR inhibition

These findings also demonstrate a differential sensitivity of pluripotent and differentiating embryonic stem cells to mTOR inhibition: rapamycin treatment induces apoptosis only in differentiating cells. These timings match the stage at which mTOR deletion become lethal: homozygous *mTOR*$^{-/-}$ embryos die at the onset of gastrulation, at around E5.5 (Gangloff et al., 2004; Murakami et al., 2004). Similarly, embryonic lethality of *S6*$^{+/-}$ mice coincides with gastrulation (Panić et al., 2006). This later study attributed the death of embryos to activation of a p53-dependent checkpoint which functions to assess whether cells carry the sufficient translational machinery to meet the high translational demands of gastrulation (Panić et al., 2006). In accordance with this, deletion of p53 in S6 heterozygous embryos permits their survival until E12.5. Therefore, the death of differentiating but not pluripotent ESCs in response to rapamycin treatment could reflect their increased need for high translational capacity, and increased sensitivity to inhibitors of translation. This could be mediated through p53 activation, which is known to be activated during gastrulation and to cause heightened sensitivity to cellular damage (Heyer et al., 2000; Laurent and Blasi, 2015).

4.3.4. Downstream of mTOR

We postulated that downstream of mTOR, elimination of defective cells could occur through repression of S6 kinase activity or induction of autophagy. This was in part because the IPA analysis revealed that the main pathways affected at the RNA expression level are involved in either translation or autophagy. Although translation changes are conferred by both 4E-BP and S6 kinase, we observed reduced phosphorylation of only S6 but not 4E-BP1 in loser cells in competition. We found that constitutive activation of S6 kinase failed to rescue elimination of tetraploid cells in competition; however, pS6 levels were also not rescued using this construct. As stable overexpression of CA S6 kinase 1 did not confer resistance of pS6 to rapamycin treatment, we concluded that compensatory mechanisms acted to degrade excess CA S6 kinase or to otherwise counteract the increase in S6 kinase activity. Future work would benefit from use of a conditional expression vector which would mimic transient expression where compensatory mechanisms are not observed. Knockdown of autophagy protein ULK1 by shRNA also did not rescue elimination of defective cells. Although this could be because autophagy induction is not important for competition, an alternate possibility is that

knockdown of the protein was not sufficient to completely disrupt autophagy. Therefore, in order to exclude a role for autophagy in defective cell elimination, autophagy levels following *Ulk1* knockdown would need to be assessed.

Unlike mTOR inhibition, repression of either S6K or 4E-BP1 alone was not sufficient to trigger cell death. One reason for this could be that both translation targets need to be inhibited before cell viability is compromised and death occurs. Alternatively, compensation of different branches of the translation network may permit cell survival when one is inhibited, as has been previously described (Chauvin et al., 2014).

4.3.5. Hyperactive mTOR signalling induces competition

Our results also demonstrated that cells with hyperactive mTOR signalling outcompete wild-type cells. How could mTOR pathway hyper-activation induce competition? It is possible that the same mechanisms which mediate loss of mTOR activity and elimination of defective cells also mediate elimination of wild-type cells by *Tsc2*$^{-/-}$ cells. This could be via competition for nutrients. For example, since *Tsc2* deletion results in increased cell glucose uptake (Duvel et al., 2010), competition for limited levels of glucose could result in death of wild-type cells when in the presence of *Tsc2*$^{-/-}$ cells. As discussed, a similar mechanism has been described to be exploited by cancer cells to 'starve' nearby T-cells (Chang et al., 2015). However, we did not observe a reduction of mTOR pathway activation in the wild-type cells when in culture with *Tsc2*$^{-/-}$ cells, suggesting that a mTOR-independent mechanism of loser cell elimination occurs during supercompetition.

Alternatively, hyperactivation of mTOR could result in death of neighbouring wild-type cells through cell non-autonomous signalling. The mTOR pathway is implicated in control of the translation of secreted factors; for example, during the SASP mTOR activation results in increased cytokine production (Herranz et al., 2015; Laberge et al., 2015; Narita et al., 2011). Here, release of cytokines has a positive growth effect on the cellular microenvironment. In other situations, mTOR hyperactivation has been shown to result in the secretion growth factors which have a negative growth effect on surrounding cells (Ding et al., 2016). For instance, *Tsc2*$^{-/-}$ MEFs secrete IGFBP5 which blocks insulin signalling in surrounding cells. Consequently, when cultured with *Tsc2*$^{-/-}$ MEFs, MCF7 cells undergo suppressed proliferation as a result of IGF signalling blockage. Due to the high sensitivity of differentiating ESCs to inhibition of cell growth pathways (as discussed below), it is possible that insulin pathway

inhibition by IGFBP5 could induce cell death, rather than simply a proliferation arrest, in wild-type ESCs. Therefore, as has been demonstrated from previous literature, mTOR hyper-activation could promote the secretion of factors which limits growth and induces death in surrounding cells. To test this hypothesis, it would be informative to investigate whether conditioned media from *Tsc2*$^{-/-}$ cells results in growth inhibition or apoptosis of wild-type cells.

Another possibility is that the high rates of glycolysis induced by *Tsc2*$^{-/-}$ cells result in the production of high levels of lactate (Duvel et al., 2010). This would have the effect of acidifying the media, and since low pH is a trigger for apoptosis, it is possible that Tsc2$^{-/-}$ cells induce wild-type death through 'lactic acidosis' (Park et al., 1999). This mechanism has been proposed to enhance invasiveness of cancer cells (Gatenby and Gawlinski, 1996), and to promote selection of mutated cells which are better able to withstand low pH in tumours (Moellering et al., 2008; Williams et al., 1999). It is not clear how *Tsc2*$^{-/-}$ cells would have more robust coping strategies for low pH. However, given that several V-ATPases, which act as vacuolar proton pumps, are mTOR gene targets (Pena-Llopis et al., 2011), it is possible that an increased ability of *Tsc2*$^{-/-}$ cells to 'pump' out protons and buffer pH could confer enhanced resistance to media acidification. Indeed, expression of V-ATPases in neutrophils is anti-apoptotic in response to cell acidification (Gottlieb et al., 1995). Therefore, *Tsc2* deletion could result in the generation of a noxious microenvironment which could result in deleterious consequences for wild-type cell viability.

A final possibility is that cell competition at this stage is triggered by relative differences in translational outputs and results in the elimination of cells with relatively lower translation levels. This theory has been put forward to account for *Minute*- and *Myc*- induced competition (Vincent et al, 2011). *Tsc2* deletion would increase cell translation and cause surrounding wild-type cells to have lower relative translation levels (Bilanges et al., 2007) See *mTOR in other models of competition* below). Although it is not known how relative fitness levels between cells could be sensed, this hypothesis would help to explain why repression of mTOR occurs during the elimination of defective cells by wild-type cells, but not during elimination of wild-type cells by *Tsc2*$^{-/-}$ cells (Fig. 4.16E).

4.3.6. Comparison of RNA silencing techniques

In this chapter, three methods of gene silencing were used to target TSC2: siRNA, shRNA and CRISPR. While optimisation of siRNA protocols yielded good knockdown after 48h, because

the approach is only transient, knockdown after this time was reduced such that knockdown effects after day 4 were subtle. We therefore sought a more stable approach to knockdown Tsc2.

In order to achieve stable knockdown over 5 days of competition, we employed a viral-mediated shRNA delivery system. Initially we tested the pGIPZ viral vector, although it had limited efficacy due to a high level of silencing during differentiation. This is a well-recognised drawback of viral-mediated gene delivery systems in mouse embryonic stem cells (Teich et al., 1977), and is attributed to two silencing mechanisms. Initially, viral promoters are recognised and blocked at the transcriptional level by DNA binding proteins (Wolf and Goff, 2009). Following this, epigenetic modifying proteins are recruited and the viral transgene becomes methylated (Schlesinger et al., 2013; Wolf and Goff, 2007). As embryonic stem cells have high levels of *de novo* methylation activity, viral transgene silencing occurs at a high rate (Cherry et al., 2000; Lei et al., 1996). The MSCV vector has been adapted to avoid recognition by transcriptional repressors and enhance binding of transcriptional promoters (Laker et al., 1998). Thus, silencing of the MSCV transgene is reduced. As well as enhancing knockdown by avoiding transcriptional repression, the MSCV vector utilises the optimised miR-E backbone which increases knockdown efficiency by enhancing pri-miRNA processing (Fellmann et al., 2013). Therefore, using an optimised viral vector with enhanced knockdown for stable RNA silencing had increased efficacy over the transient siRNA approach.

4.3.7. mTOR in other models of competition

While a direct role for mTOR in competition has not been described, many triggers of competition are involved in translation. For example, the first genes shown to instigate the loser phenotype upon mutation were the *Minute* genes, which cause cells to have defects in translation due to haploinsufficiency of ribosomal subunits (Morata and Ripoll, 1975). Conversely, increasing cell translation through overexpression of *Myc* is sufficient to drive competitive interactions (Moreno and Basler, 2004; de la Cova et al., 2004). Given the crucial role in regulation of translation by the mTOR pathway, it is possible that mosaic levels of translational output induce competition (Fig. 4.19).

Previous studies in *Drosophila* have reported that *Tsc1/2* deletion does not rescue the elimination of *Minute* cells in imaginal wing discs (Tyler et al., 2007). Possibly, this is because the translation defect is downstream of mTOR, and so hyper-activation of the pathway would

be unable to rescue translation output induced by deletion of a ribosomal gene (Fig. 4.19). To test this, it would be interesting to determine whether mutations which do not directly affect translation output, such as those important for polarity or signalling, show reduced mTOR activity in competition, and are rescued by *Tsc2* deletion. Furthermore, it would be important to ascertain whether *Bmpr1a*$^{-/-}$ or 4n cells show reduced translation in competition, and whether rescue of this is sufficient to rescue their elimination. An alternative option is that the role of mTOR in loser cell elimination is not conserved in *Drosophila*, or that it is a specific feature of competition during mouse gastrulation.

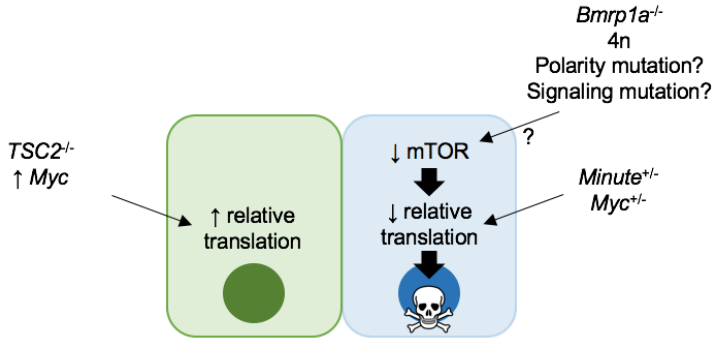


Figure 4.19: Model for how differential activation of mTOR could fit into other competition models.

Possibly, the relative reduction in translation instigated by mTOR repression triggers elimination of cells. *Minute*$^{+/-}$ and *Myc*$^{+/-}$ have defective translation, explaining why *Tsc2* deletion does not rescue *Minute*$^{+/-}$ cell loss. This model could also explain super-competition driven by *Tsc2* deletion or Myc overexpression: both genetic alterations would result in translation gradients between cells. Since the wild-type cells would have relatively lower translation rates these would be eliminated.

Chapter 5: p53 mediates cell competition during early mouse development

5.1. Introduction

5.1.1. Diverse roles of p53

p53 is a protein which responds to a plethora of stress signals and acts to induce cell cycle arrest or apoptosis (Vousden and Prives, 2009). The ability of the protein to halt cell cycle progression or induce cell death in the face of DNA damage, hypoxia, nutrient deprivation, and hyper-proliferation renders it a potent tumour suppressor which is deleted or mutated in half of all human cancers (Olivier et al., 2010). These canonical functions of p53 are largely elicited through its activity as a transcription factor, and most p53 mutations found in human tumours are in the protein's DNA-binding domain (Olivier et al., 2010; Villunger et al., 2003). However, cytoplasmic p53 also induces apoptosis through mitochondrial permeabilisation (Green and Kroemer, 2009). On top of these functions as 'guardian of the genome', p53 has been implicated in a diverse and expanding range of other cellular process, including metabolism, reprogramming, EMT, and immune regulation (Vousden and Prives, 2009). Furthermore, when expressed at basal levels the protein has a variety of more subtle roles including in stem cell renewal (Liu et al., 2009; Meletis et al., 2006), cell migration (Godar et al., 2008), and blastocyst implantation (Hu et al., 2007).

As expected for a protein which is involved in such a wide variety of cell processes, there are numerous modifications which alter the activity of p53. Under basal conditions, p53 activity is kept in check by mouse double minute protein 2 (Mdm2) which ubiquitinates the C-terminus of p53 and targets it for degradation (Piette et al., 1997). Since Mdm2 is itself is a target of p53, this regulation functions as part of a negative feedback loop. Upon triggers such as DNA damage, upstream pathways act to either block Mdm2-mediated p53 ubiquitination or to stabilise p53 directly through post-translational modifications (Kruse and Gu, 2009). These post-translational modifications are diverse and include phosphorylation, acetylation, methylation, glycosylation and farnesylation, to name a few (Kruse and Gu, 2009).

The downstream activity of p53 is also remarkably complex and different subsets of genes are activated or repressed depending on whether p53 activated basally, acutely or chronically (Kirschner et al., 2015). Understanding how p53 selects its targets in response to different

conditions is a subject of ongoing research (Kruiswijk et al., 2015). However, it is likely to depend on p53 post-translational modifications, epigenetic alterations, the availability of binding partners in different conditions, and the myriad indirect transcriptional changes which occur in response to modulation of direct p53 targets (Kruiswijk et al., 2015).

5.1.2. p53 as a repressor of the mTOR pathway

A more recently described function of p53 is in the modulation of mTOR pathway activity. Activation of p53 in response to genotoxic stress has been shown to promote the transcriptional upregulation of negative regulators of the mTOR pathway such as Pten, Tsc2 and Ampkβ1 (Feng et al, 2007). Similarly, the protein products of p53 target genes *Sestrin1* and *Sestrin2* (*Sesn1/2*) inhibit mTOR pathway activity through phosphorylation of AMPK which leads to the activation of TSC2 (Budanov and Karin, 2008). Finally, p53-target *Regulated in development and DNA damage 1* (*Redd1/ Ddit4*) also acts to inhibit mTOR pathway activity (Cam et al., 2014; Kenzelmann Broz et al., 2013; Vadysirisack et al., 2011).

5.1.3. p53 in development

Since *p53*-null mice develop normally, it was initially thought that p53 is not required for development (Donehower et al., 1992). However, p53 is expressed during embryogenesis (Schmid et al., 1991) and activation of p53 has been shown to trigger differentiation in ESCs (Lin et al., 2005; Shigeta et al., 2013), although it is dispensable for differentiation *in vivo* (Shigeta et al., 2013). Recently these discrepancies were aligned by the finding that there is functional redundancy between p53 family members p53, p63 and p73 during early mouse development (Wang et al., 2017). Deletion of all three family members prevents mesendoderm induction, indicating that p53 has roles in directing lineage specification during development (Wang et al., 2017). Supporting this, deletion of p53 in *Xenopus laevis*, which only have one p53-family gene, fail to gastrulate due to lack of mesendoderm differentiation (Cordenonsi et al., 2003; Sasai et al., 2008).

5.1.4. Aims

In the previous chapter, our results revealed that defective cell elimination occurs as a result of repression of the mTOR pathway in our loser cell lines. Our next aim was to determine what causes the repression of the mTOR pathway in loser cells during competition. Specifically, we wanted to investigate whether mTOR repression in loser cells occurs as a result of competition

for a limited supply of extracellular factors, or alternatively whether mTOR inhibition occurs as a result of activation of an internal molecular repressor.

5.2. Results

5.2.1. Competition does not seem to be mediated by limitation of shared factors in the media

Since mTOR integrates a variety of extracellular signals, we aimed to understand whether the reduced levels of mTOR pathway activation observed in defective cells occurs as a result of competition for a limited pool of nutrients. Amino acids provide a major source of mTOR-activating signals. We therefore supplied the media of competing wild-type and *Bmpr1a*$^{-/-}$ cells with 10x the normal level of either essential amino acids (EAA) or non-essential amino acids (NEAA) and measured cell ratios. However, this rescued neither cell ratios nor pS6 levels, indicating that *Bmpr1a*$^{-/-}$ cells are not competing with wild-type cells for limited levels of amino acids (Fig. 5.1). This result will need to be confirmed by independent repeats.

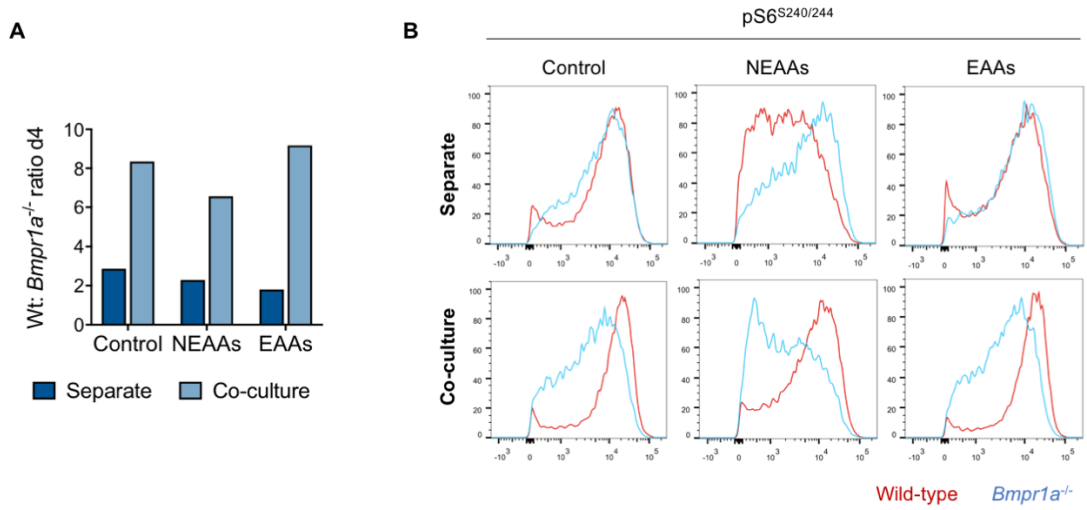


Figure 5.1: Supplying media with additional amino acids does not rescue competition.
A. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured alone and together with either control media, media plus 10x concentration of non-essential amino acids (NEAAs) or media plus 10x concentration of essential amino acids (EAAs). The ratio of wild: *Bmpr1a*$^{-/-}$ cells was calculated after 4 days in N2B27, and pS6 levels were determined by flow cytometry after 3 days in N2B27 (B). n=1.

The insulin sensing pathway is also a major upstream input into the mTOR pathway. To assess whether reduced insulin signalling could be responsible for the reduced levels of mTOR pathway activity in loser cells, we attempted to hyperactivate the pathway by infecting *Bmpr1a*$^{-}$

$^{-/-}$ cells with viruses expressing shRNAs targeting Pten, an inhibitor of the insulin-sensing pathway. Although we observed good knockdown of PTEN using these shRNAs (Fig. 5.2A), we did not observe a rescue in pS6 levels or *Bmpr1a*$^{-/-}$ cell number during competition (Fig. 5.2B,C). Furthermore, exogenous application of insulin to the media did not alter *Bmpr1a*$^{-/-}$ cell numbers (Fig. 5.2D). We conclude that *Bmpr1a*$^{-/-}$ cells are not competing with wild-type cells for limited levels of insulin.

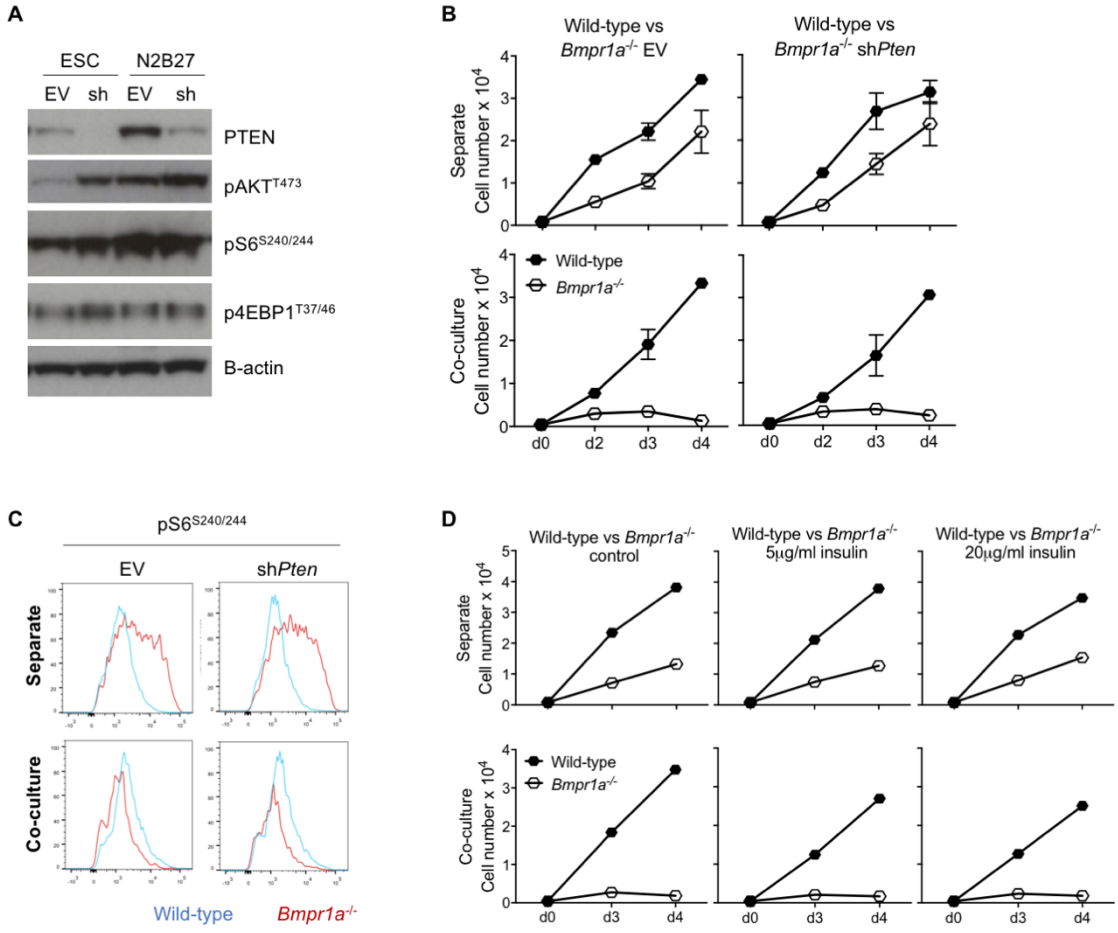


Figure 5.2: Increasing insulin pathway activation or supplying media with additional insulin does not rescue competition.

A. *Bmpr1a*$^{-/-}$ cells were infected with MSCV vectors expressing PTEN-targeting shRNAs, and knockdown of PTEN and activation of insulin sensing pathway (pAKT) in pluripotent and differentiating cells was confirmed by Western blot. B. Growth curves of wild-type and *Bmpr1a*$^{-/-}$ cells containing empty (EV) and *Pten*-targeting shRNAs were assessed over 4 days in culture. Error bars denote SEM; n=2. C. pS6 staining in wild-type and *Bmpr1a*$^{-/-}$ cells with and without Pten knockdown in separate and co-culture. D. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured separately and together in the presence of control media and media with added 5mg/ml or 20mg/ml insulin. n=1.

We next performed two experiments which aimed to assess whether cells could be competing for a limited pool of nutrients from a less biased perspective. First, we hypothesised that in order to be outcompeted by wild-type cells for shared extracellular factors, *Bmpr1a*$^{-/-}$ cells would need to have a disadvantage in up-taking or transducing environmental signals. Therefore we aimed to identify whether defective cells have a delay in responding to mTOR-activating stimuli in non-competitive conditions. We starved wild-type and *Bmpr1a*$^{-/-}$ cells for 1 hour to abolish mTOR pathway activity and then re-supplied cells with fresh media and measured the return of mTOR activity at different time-points. We found that both wild-type and *Bmpr1a*$^{-/-}$ cells responded at similar times to refeeding (Fig. 5.3A). We therefore conclude that *Bmpr1a*$^{-/-}$ cells do not have a delay in responding to the extracellular environment, indicating that they would not have a disadvantage in up-taking or transducing these signals compared to wild-type cells in co-culture. Secondly, we reasoned that if cells were competing for a limited pool of extracellular nutrients and the reduced mTOR activation in the *Bmpr1a*$^{-/-}$ cells was due to their reduced ability to access these nutrients, then replenishing the culture with fresh media should rescue reduced mTOR levels. Our starvation experiments revealed that 30 minutes fresh media is sufficient to re-activate the mTOR pathway in starved cells (Fig. 5.3A). However, refeeding competing cells for 30 minutes did not fully restore pS6 levels in competing *Bmpr1a*$^{-/-}$ cells (Fig. 5.3B). Together, these results suggest that the reduced mTOR activity in *Bmpr1a*$^{-/-}$ is not due to a reduced ability to take in nutrients from their environment, but rather could represent activation of molecular inhibition during competition (Fig. 5.3B).

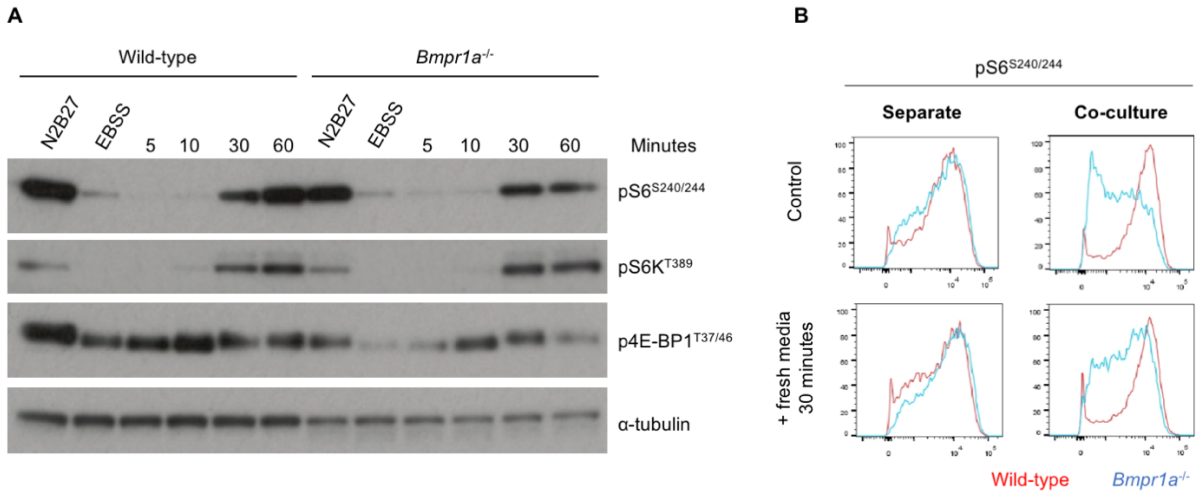


Figure 5.3: Wild-type and *Bmpr1a*$^{-/-}$ cells respond at similar times to mTOR-activating stimuli separate culture but not co-culture.

A. Wild-type and *Bmpr1a*$^{-/-}$ cells cultured separately were starved using EBSS media for 30 minutes, supplied with fresh media, and protein was collected at different time-points following refeeding and

mTOR activation level was assessed by Western blot. n=2. B. Wild-type and *Bmpr1a*$^{-/-}$ cells in separate and co-culture were supplied with fresh media for 30 minutes and pS6 levels were determined by flow cytometry. n=2.

5.2.2. Defective cells have elevated levels of p53

Since the above data indicates that differential mTOR levels are not resulting from competition for shared factors in the media, we took a systematic approach to identify changes in gene expression which occur in *Bmpr1a*$^{-/-}$ cells which could account for reduced levels of mTOR pathway activity. When comparing gene expression profiles of co-cultured *Bmpr1a*$^{-/-}$ and wild-type cells using IPA (Fig. 4.1), p53 was identified as the top upstream regulator ranked according to *p*-value (Fig. 5.4).

Upstream regulator	*p*-value
TP53	6.73E-33
MYC	2.59E-18
L-dopa	3.87E-18
beta-estradiol	1.93E-17
ERBB2	2.55E-17

Figure 5.4: p53 is top upstream regulator in IPA analysis.

IPA analysis comparing gene expression changes in *Bmpr1a*$^{-/-}$ cells vs wild-type cells in co-culture after 3 days in N2B27 revealed p53 as the top upstream altered component when ranked by *p*-value.

To identify whether p53 protein levels are different between wild-type and loser cells, we performed immunofluorescence staining of p53 in wild-type and *Bmpr1a*$^{-/-}$ or 4n cells cultured alone and together after 3 days in N2B27. This revealed that defective cell types had elevated levels of nuclear p53 compared to wild-type cells in both separate and co-culture (Fig. 5.5). These results confirm our IPA analysis and suggest that elevated p53 could be a general marker of reduced cell fitness.

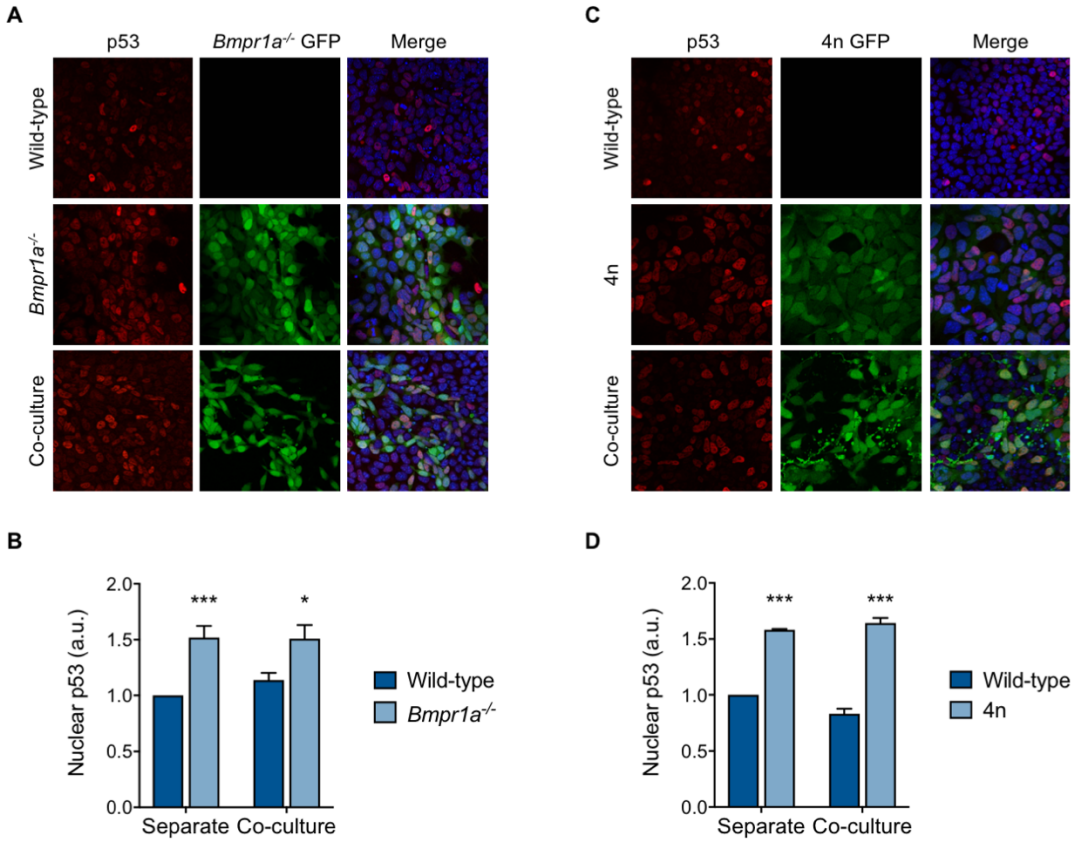


Figure 5.5: p53 is upregulated in loser cells.

A. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured separately and together for 3 days in N2B27 and p53 levels were assessed by immunofluorescence and quantified (B). C. Wild-type and 4n cells were cultured separately and together for 3 days in N2B27 and p53 levels were assessed by immunofluorescence and quantified (B). *, $p<0.05$; ***, $p<0.001$, 2-way ANOVA followed by Tukey's post-hoc test. Error bars denote SEM. n=3.

5.2.3. p53 inhibits the mTOR pathway

Since p53 has been described to act upstream of mTOR to repress its activity (Akeno et al., 2015; Feng and Levine, 2010), we next asked whether p53 acts as an upstream inhibitor of the mTOR pathway in our system of differentiating ESCs. First, we treated *Bmpr1a*$^{-/-}$ cells with p53 activator Nutlin-3a. This led to an decrease in mTOR pathway activity (Fig. 5.6A,B). Next, we infected wild-type cells with shRNAs targeting *p53*. Knockdown of p53 resulted in an increase in mTOR pathway activity (Fig. 5.6C). p53 has also been described to act as a downstream effector of mTOR signalling (Mungamuri et al., 2006). To investigate whether this is the case in our cells, we analysed p53 levels following mTOR inhibition (using mTOR inhibitor rapamycin) or hyperactivation (by deletion of TSC2 in wild-type cells). We observed

that p53 levels were unaffected by manipulating mTOR activity by these methods (Fig. 5.6D,E). Therefore we conclude that in differentiating mESCs, p53 acts upstream of mTOR to inhibit mTOR pathway activity.

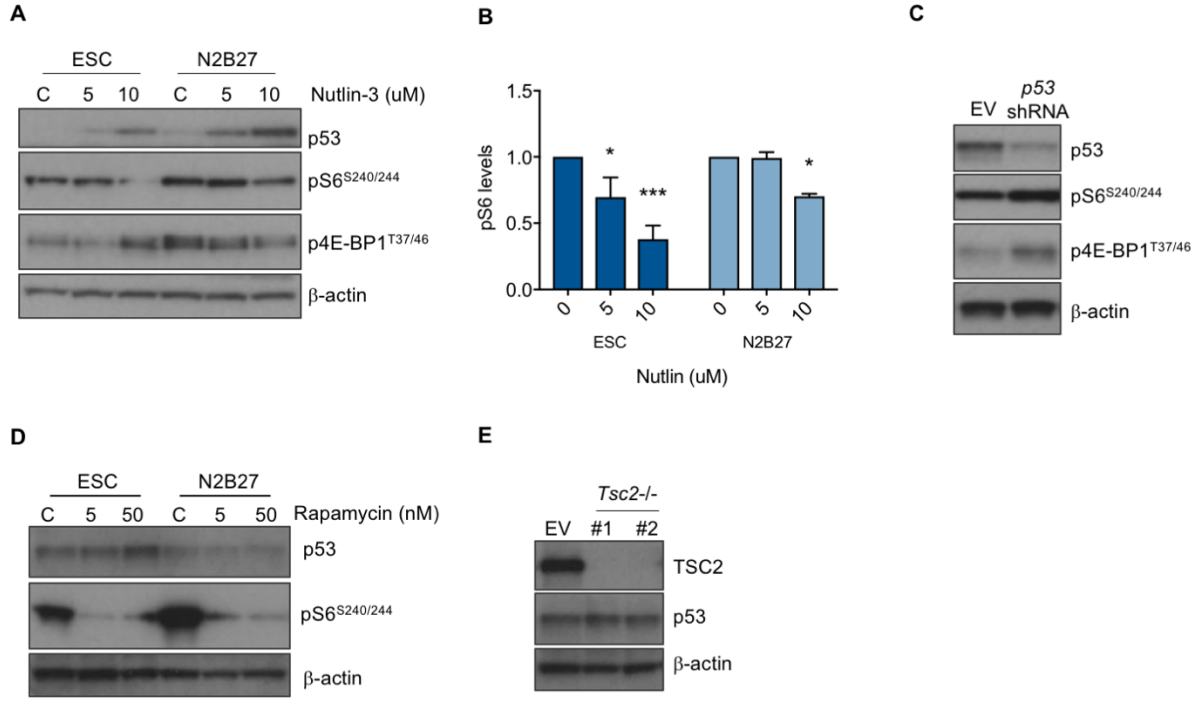


Figure 5.6: p53 acts upstream of the mTOR pathway to inhibit its activity.

A. *Bmpr1a*$^{-/-}$ cells were treated with p53 activator Nutlin-3a and activation levels of mTOR pathway components were assessed by Western blot and quantified (B). C. mTOR pathway activity was assessed in control (EV) and p53-depleted (*p53* shRNA) wild-type cells by Western blot. D. Wild-type cells were treated with rapamycin and levels of p53 were assessed by Western blot. E. Levels of p53 were assessed in wild-type and *Tsc2*$^{-/-}$ cell lines. *, $p<0.05$; ***, $p<0.001$, 2-way ANOVA followed by Tukey's post-hoc test. Error bars denote SEM. n=3.

5.2.4. Defective cell elimination and loss of mTOR activity requires p53

Since our data has demonstrated that p53 is upregulated in our loser cell lines and that it can inhibit mTOR activity, we next wanted to understand whether p53 could be acting upstream of mTOR during competition. We used siRNAs to knockdown p53 in competing wild-type and *Bmpr1a*$^{-/-}$ cells to around 15% of controls (Fig. 5.7A,B). Knockdown of p53 resulted in a partial rescue of *Bmrp1a*$^{-/-}$ cell growth (Fig. 5.7C,D), and a rescue of pS6 levels (Fig. 5.7E,F). These effects are independent of differentiation as expression of cell surface pluripotency marker CD31 was lost in cells transfected with both control and p53 siRNAs (Fig. 5.7G).

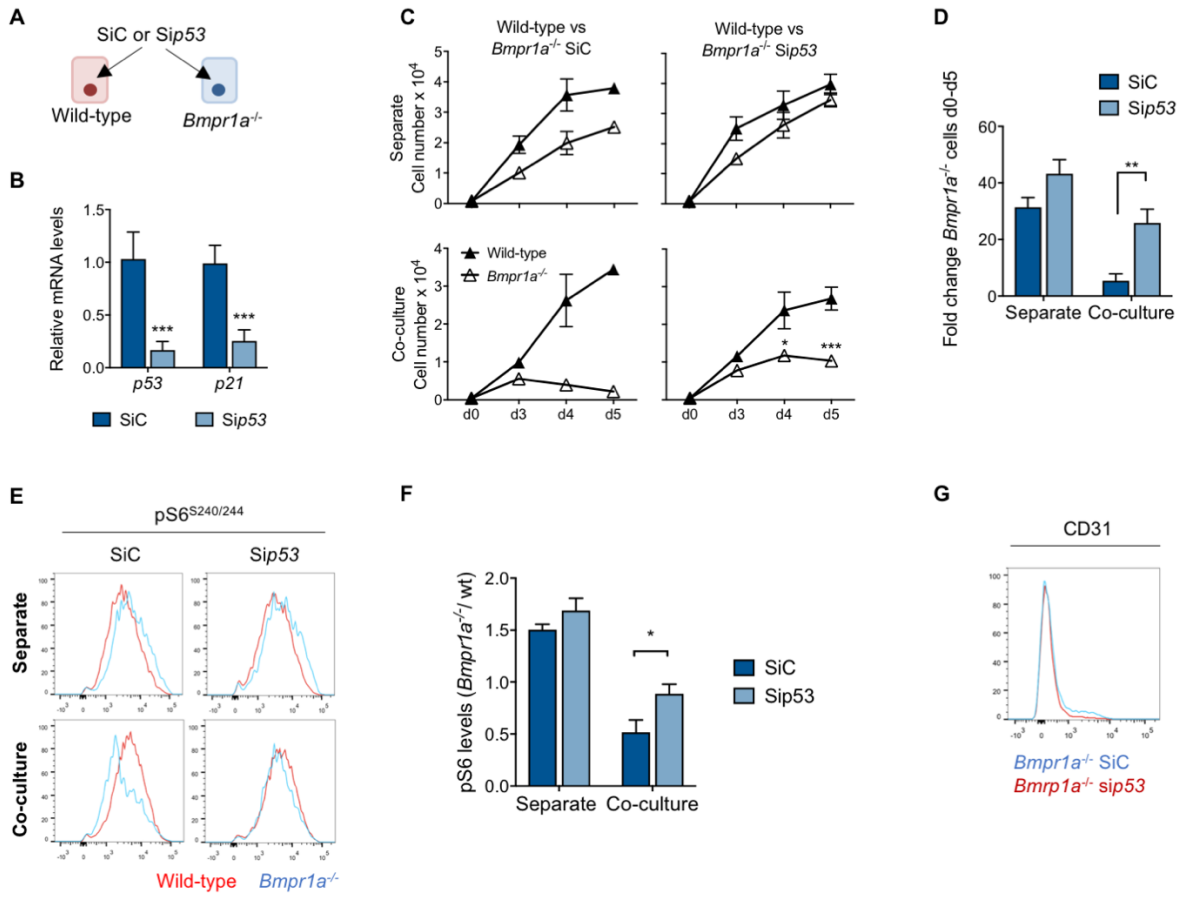


Figure 5.7: Knockdown of p53 partially rescues *Bmpr1a*$^{-/-}$ growth and pS6 levels in competition.
A. Both wild-type and *Bmpr1a*$^{-/-}$ cells are subjected to control or p53 knockdown. B. Efficacy of p53 knockdown was assessed by analysing p53 and target p21 mRNA levels. C. Growth curves of wild-type and *Bmpr1a*$^{-/-}$ cells in control and p53-depleted cells were assessed over 5 days in separate and co-culture. D. Fold change in control and p53-depleted *Bmpr1a*$^{-/-}$ cells in separate and co-culture from d0-d5. E. Levels of pS6 were assessed by flow cytometry in wild-type and *Bmpr1a*$^{-/-}$ cells in separate and co-culture following control or p53-knockdown and quantified (F). G. Levels of pluripotency marker CD31 in control and p53-depleted *Bmpr1a*$^{-/-}$ cells was assessed by flow cytometry. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, student's t-test (B) or 2-way ANOVA followed by Tukey's (D,F) post-hoc test. Error bars denote SEM. n=3.

We hypothesised that the partial rescue of *Bmpr1a*$^{-/-}$ cell growth in the presence of wild-type cells following treatment with p53 siRNA was due to the transient nature of the knockdown. To test if complete abrogation of p53 in loser cells led to a more dramatic rescue of defective cell elimination, we generated *Bmpr1a*$^{-/-}$*; p53*$^{-/-}$ cells using CRISPR. We found that deletion of p53 not only rescued the elimination of BMP defective cells in competition with wild-type cells, but also led them to induce the death of wild-type cells in co-culture conditions (Fig.

5.8A,B). Furthermore, p53 deletion prevented a down-regulation of mTOR activity in loser cells (Fig. 5.8C). Deletion of p53 did not prevent ESC differentiation (Fig. 5.8D) indicating that the above results are not due to p53 mutation maintaining cells in a pluripotent state. These results suggest that elevated levels of p53 in defective cells are important for driving competitive interactions between cells and therefore for repressing mTOR pathway activity.

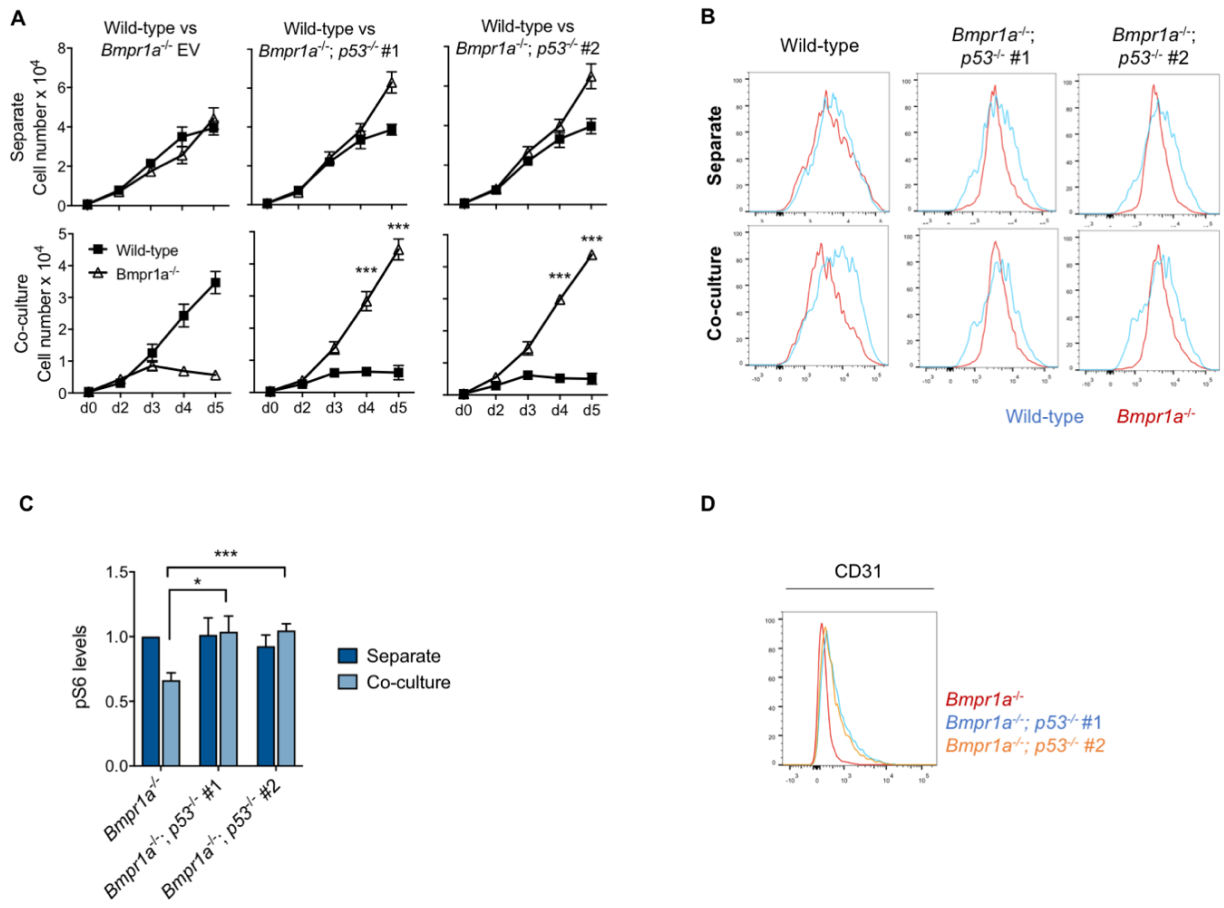


Figure 5.8: Deletion of p53 in *Bmpr1a*$^{-/-}$ rescues their elimination and loss of pS6.

A. Competition assay was performed between wild-type and control (EV) or *p53*-null *Bmpr1a*$^{-/-}$ cells over 5 days. B. Levels of pS6 were assessed by flow cytometry at d3 in competition between wild-type and control or p53-null *Bmpr1a*$^{-/-}$ cells and quantified (C). D. Levels of pluripotency marker CD31 in control and *p53*-null *Bmpr1a*$^{-/-}$ cells were assessed by flow cytometry. *, p<0.05; ***, p<0.001, 2-way ANOVA followed by Tukey's post-hoc test (C). Error bars denote SEM. n=3.

Next we aimed to identify if p53 is also important in our 4n model of cell competition. Knockdown of p53 by siRNA reduced mRNA levels to ~33% controls and partially rescued 4n cell elimination (Fig. 5.9A-D). Furthermore, reducing p53 levels rescued pS6 in 4n cells (Fig. 5.9E-F). As 4n cells differentiated similarly to control cells we concluded that this effect

is not due to inhibition of differentiation (Fig. 5.9G). In summary our results suggest that the increased levels of p53 in loser cells is important for their elimination and for the reduction of mTOR pathway activity.

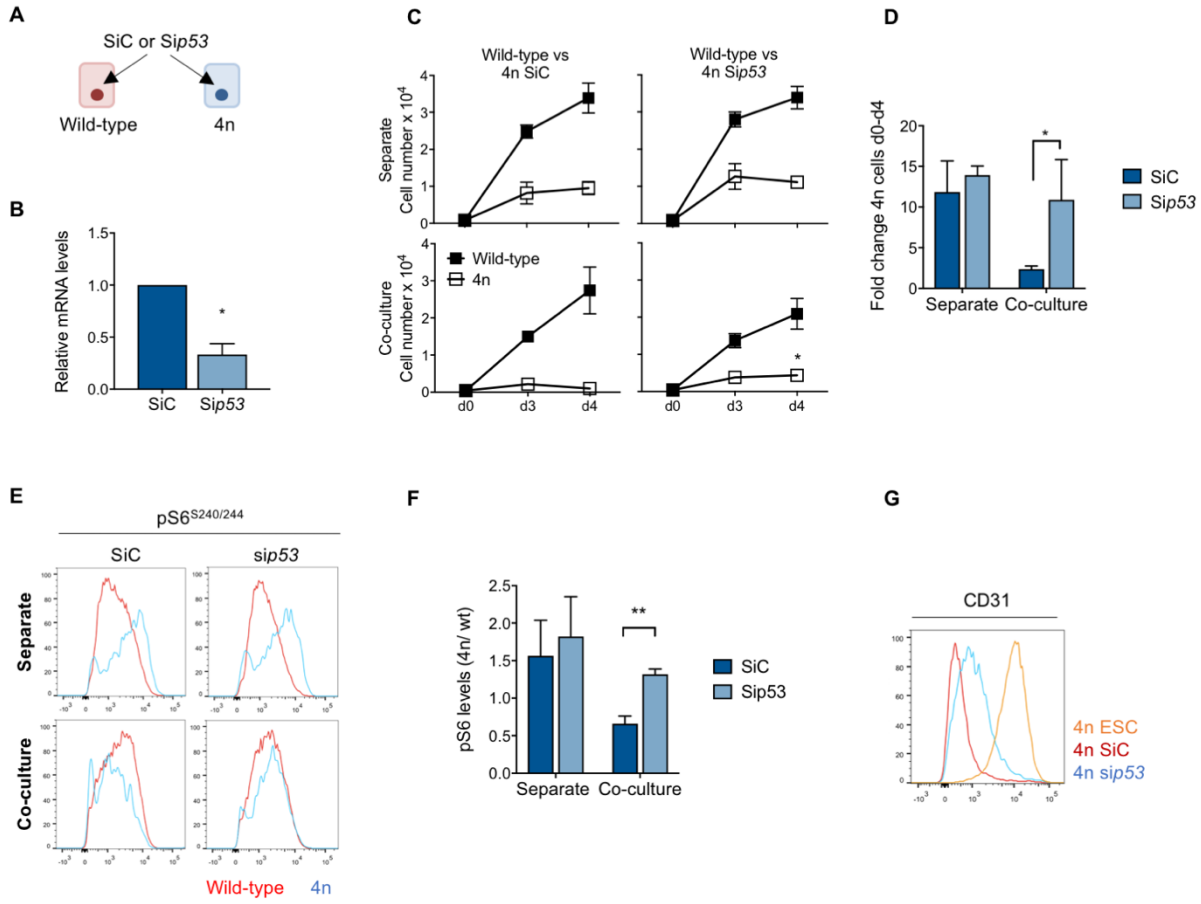


Figure 5.9: Knockdown of p53 partially rescues 4n growth and pS6 levels in competition.

A. Both wild-type and 4n cells are subjected to control or p53 knockdown. B. Efficacy of p53 knockdown was assessed by analysing p53 mRNA levels. C. Growth curves of wild-type and 4n cells in control and p53-depleted cells were assessed over 4 days in separate and co-culture. D. Fold change in control and p53-depleted 4n cells in separate and co-culture from d0-d5. E. Levels of pS6 were assessed by flow cytometry in wild-type and 4n cells in separate and co-culture following control or p53-knockdown and quantified (F). G. Levels of pluripotency marker CD31 in control and p53-depleted 4n cells was assessed by flow cytometry. *, $p<0.05$; **, $p<0.01$, student's t-test (B,C) or 2-way ANOVA followed by Tukey's (D,F) post-hoc test. Error bars denote SEM. n=3.

5.2.5. p53 activity is altered specifically during competition

Our data suggest that p53 acts upstream of the mTOR pathway to inhibit its activity in *Bmpr1a*$^{-/-}$ cells during competition. We next aimed to understand why p53 expression is upregulated in loser cells in both separate culture and co-culture, but mTOR is only repressed in co-cultures.

Since we did not observe a further upregulation of p53 in competing loser cells compared to cells in separate culture (Fig. 5.5), we investigated whether there is evidence that p53 could instead undergo a change in activity in co-culture to repress mTOR. Changes in p53 activity determine the subset of targets regulated (Bieging et al., 2014; Kirschner et al., 2015; Kruiswijk et al., 2015) and for this reason we investigated the expression of p53 target genes *Tsc2*, *Pten*, *Ampkβ1*, *Redd1* (*Ddit4*) and *Sesn1-2*, which encode proteins that repress mTOR activity (Berkers et al., 2013; Budanov and Karin, 2008; Ellisen et al., 2002). We observed that in contrast to *p21*, a canonical p53 target gene that was up-regulated in defective cells in both separate and co-culture conditions (Fig. 5.10A), *Redd1* and *Sesn1* were up-regulated in defective cells only in the co-culture condition (Fig. 5.10B). To test the involvement of Redd1 we treated cells with translation inhibitor cyclohexamide, which has been described to increase mTOR activity by preventing expression of Redd1 (Kimball et al., 2008). Culturing cells with cyclohexamide for 15 minutes restored pS6 levels (Fig. 5.10C). Although it should be noted that cyclohexamide has also been proposed to increase pS6 levels by raising concentrations of internal amino acids (Beugnet et al., 2003), this finding provides some support for a role of REDD1 in mediating repression of mTOR in competing loser cells. In summary, our data suggest that the confrontation of wild-type and defective cells may lead to a change in p53 activity which leads to a repression in mTOR signalling. Alternatively, it is also possible that a p53-independent pathway becomes activated during competition and leads to the repression of mTOR and elimination of loser cells.

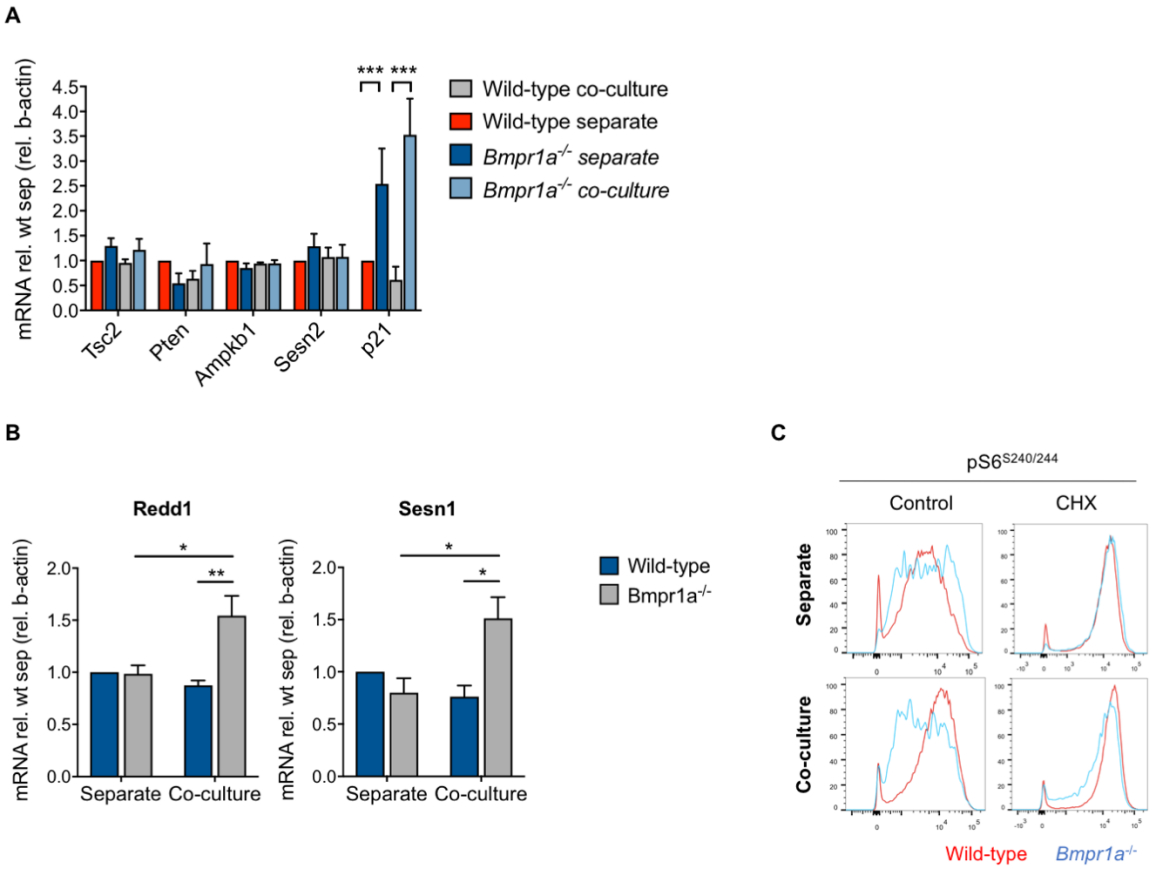


Figure 5.10: p53-target genes Redd1 and Sesn1 upregulated in *Bmpr1a*$^{-/-}$ cells in competition.
A, B. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured together and separate for three days in N2B27, sorted by FACS, and levels of p53-target genes were assessed by qPCR. C. Levels of pS6 in wild-type and *Bmpr1a*$^{-/-}$ cultured separately and together following cyclohexamide (CHX) treatment were assessed by flow cytometry. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$, students t-test followed by Holm-Sidak post-hoc test (A); 2-way ANOVA followed by Sidak's (B) post-hoc test. Error bars denote SEM. n=3.

We next aimed to understand what causes p53 activation in loser cells, or what could lead to its change in activity in co-culture. First, we stained cells for γH2AX to identify whether they showed signed of DNA damage during competition, as this protein acts as a marker of double-strand breaks. However, *Bmpr1a*$^{-/-}$ cells did not stain positively for γH2AX when in co-culture with wild-type cells (Fig. 5.11A). Next, we used hypoxyprobe to identify whether p53 activation could be caused by hypoxia, as previously described (Sermeus and Michiels, 2011). Culturing cells in hypoxic conditions led to an increase in hypoxyprobe staining (Fig. 5.11B). However, there were no differences in hypoxia levels between wild-type and *Bmpr1a*$^{-/-}$ in co-culture after 3 days in N2B27 (Fig. 5.11C). These results suggest that p53 is not activated by DNA damage or hypoxia.

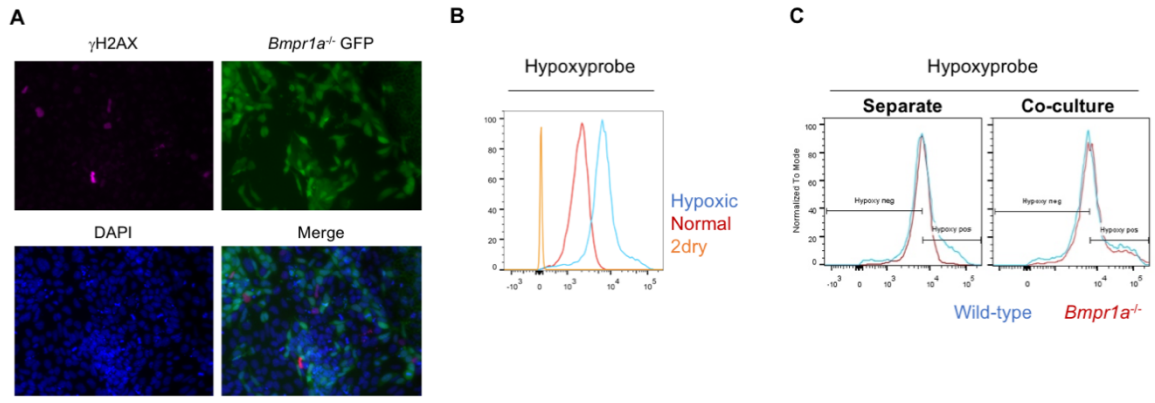


Figure 5.11: Investigating stress pathways upstream of p53.

A. Wild-type and *Bmpr1a*$^{-/-}$ cells were cultured together and separately for three days in N2B27 and levels of DNA damage marker were assessed by immunofluorescence. n=1. B. Wild-type cells were cultured in normal or hypoxic conditions prior to staining with hypoxia marker hypoxyprobe and analysing stain by flow cytometry to check marker efficacy. C. Levels of hypoxia in wild-type and *Bmpr1a*$^{-/-}$ cells cultured separately and together for three days in N2B27 were assessed by flow cytometry using hypoxyprobe. n=2.

5.2.6. Differences in p53 activity are sufficient to cause cell competition

Deletion of p53 in the *Bmpr1a*$^{-/-}$ cells resulted in overgrowth of *Bmrp1a*$^{-/-}$; *p53*$^{-/-}$ cells at the expense of wild-type cells, a finding which suggested that supercompetition occurs (Fig. 5.8). To analyse p53-mediated supercompetition further, we deleted *p53* in wild-type cells using CRISPR and performed competition assays with unaltered wild-type cells (5.12A). Initially we selected two *p53*$^{-/-}$ clones with absent levels of p53 (Fig. 5.12B). While *p53*$^{-/-}$ cells grew at similar rates to wild-type cells in separate culture, they induced the elimination of wild-type cells in co-culture (at day 4 ~90% of cells were *p53*$^{-/-}$; 5.12C,D). The proportion of cleaved caspase-3-positive wild-type cells increased at day 4 in the co-cultures compared to the separate, indicating that their elimination was at least in part mediated by apoptosis (Fig. 5.12E). Furthermore, wild-type and *p53*$^{-/-}$ cells stained similarly for pluripotency marker CD31 indicating that these effects are independent of differentiation (Fig. 5.12F).

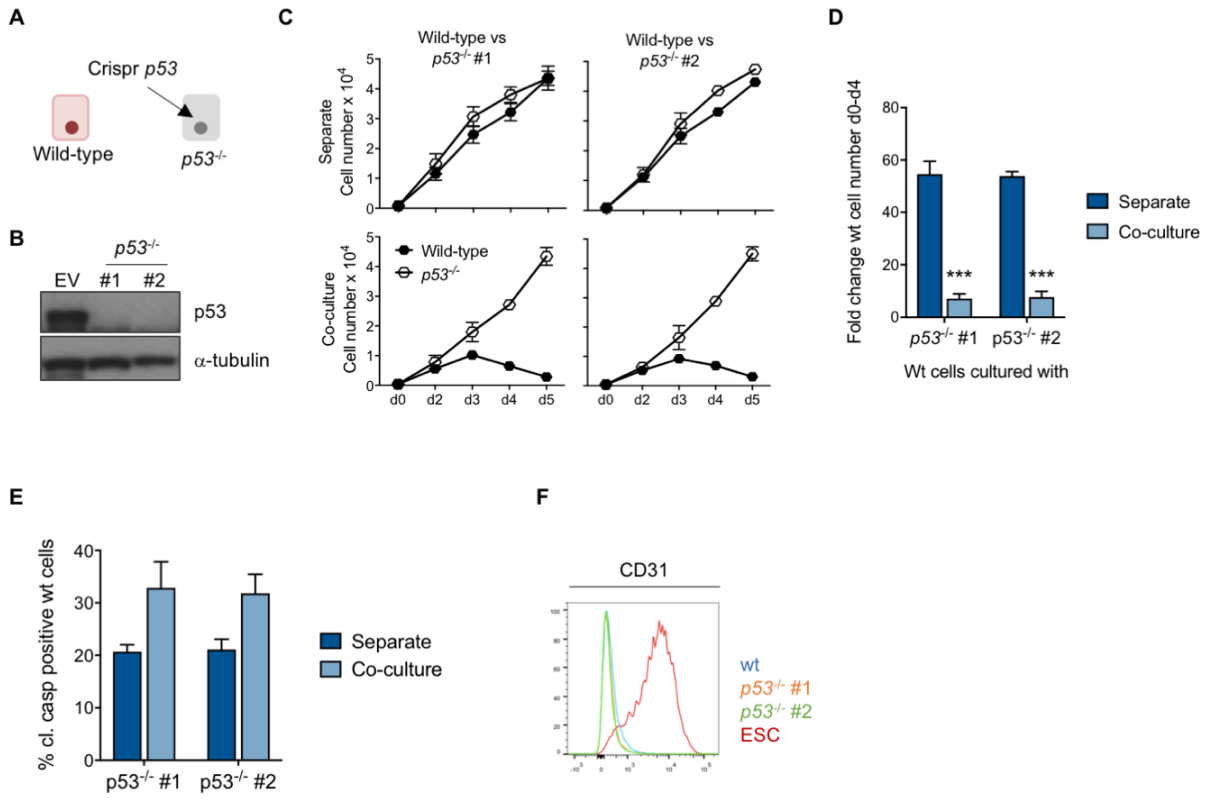


Figure 5.12: Supercompetition is triggered by differences in p53.

A. Competition assays were performed between wild-type and *p53*-null cells which were generated by CRISPR. B. Two clones of *p53*-null cells were selected for competition analysis. C. Growth curves of wild-type and $p53^{-/-}$ cells in separate and co-culture were assessed over 5 days in N2B27. D. Fold change in wild-type cell number between d0-d4 when cells are cultured alone and with $p53^{-/-}$ cells. E. Levels of cleaved caspase-3 in wild-type cells cultured alone or with $p53^{-/-}$ cells were assessed by flow cytometry. F. Levels of pluripotency marker CD31 were assessed in differentiated wild-type and $p53^{-/-}$ cells and pluripotent wild-type cells by flow cytometry. ***, $p<0.001$, 2-way ANOVA followed by Sidak's post-hoc test. Error bars denote SEM. n=3.

We next aimed to assess whether wild-type cell elimination in the presence of p53-null cells was caused by a repression of mTOR pathway activity. We stained competing wild-type and *p53*-null cells for pS6 at day 3 in competition. To our surprise, we did not observe reduced levels of mTOR pathway activity in wild-type cells when in co-culture with $p53^{-/-}$ cells, suggesting that either the timings of p53-mediated supercompetition are different to defective-cell competition, or that p53-mediated supercompetition is independent of changes in mTOR pathway activity (Fig. 5.13A). To investigate further, we performed competition assays of $p53^{-/-}$ and $Tsc2^{-/-}$ cells, which we had also found to act as supercompetitors (Fig. 4.13B). We found that co-culture of $p53^{-/-}$ and $Tsc2^{-/-}$ cells did not trigger elimination of either cell type. We can

conclude from these results that either elimination of wild-type cells in the presence of *p53*$^{-/-}$ cells requires repression of mTOR which is not detectable at day 3, or that both *p53*$^{-/-}$ and *Tsc2*$^{-/-}$ cells are of equal competitive status.

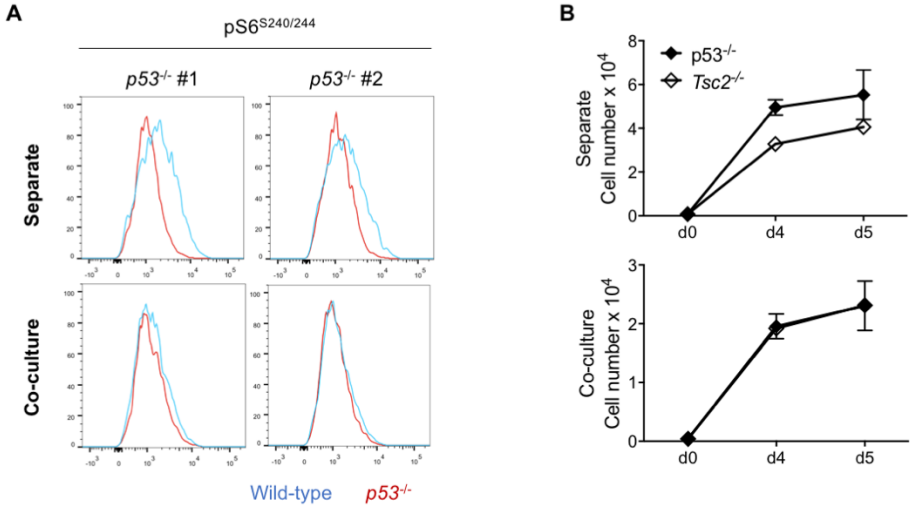


Figure 5.13: Investigating mTOR pathway in p53-mediated super-competition.

A. Wild-type and *p53*$^{-/-}$ cells were cultured together and separately for 3 days in N2B27 and pS6 levels were determined by flow cytometry. B. *p53*$^{-/-}$ and *Tsc2*$^{-/-}$ cells were cultured together and separately and growth curves were calculated at d0, d4 and d5. n=3.

5.2.7. p53 differences drive super-competition *in vivo*

Since loss of p53 led to highly aggressive behaviour against wild-type cells in *in vitro* culture, we next asked if altering competitive interactions in this way had any effect on development. We therefore analysed the behaviour of *p53*$^{-/-}$ cells in chimeras. Between E5.0 and E5.5 the mouse embryo cavitates to generate a cup-shaped epiblast that is a columnar epithelium surrounding the pro-amniotic cavity (Rivera-Perez and Hadjantonakis, 2014). Analysis of E6.5 chimeras revealed a striking result: we observed that in contrast to *p53*$^{-/-}$ embryos that cavitate normally (Bedzhov and Zernicka-Goetz, 2014) and survive until adulthood (Donehower et al., 1992), in chimeras with wild-type cells, *p53*$^{-/-}$ cells overgrow into the embryonic and/or extra-embryonic cavities (Fig. 5.14A). Furthermore, the wild-type cells surrounding the *p53* mutant cells displayed noticeable signs of apoptosis (Figure 5.14A). To understand the consequences of this overgrowth we studied E9.5 chimeric embryos and found that these displayed a wide range of developmental defects, including a variety of tissue overgrowths, failure of normal heart looping, abnormal epithelial structures and exencephaly (Fig. 5.14B,C). These results

suggest that p53-driven super-competition is sufficient to severely disrupt normal morphogenesis.

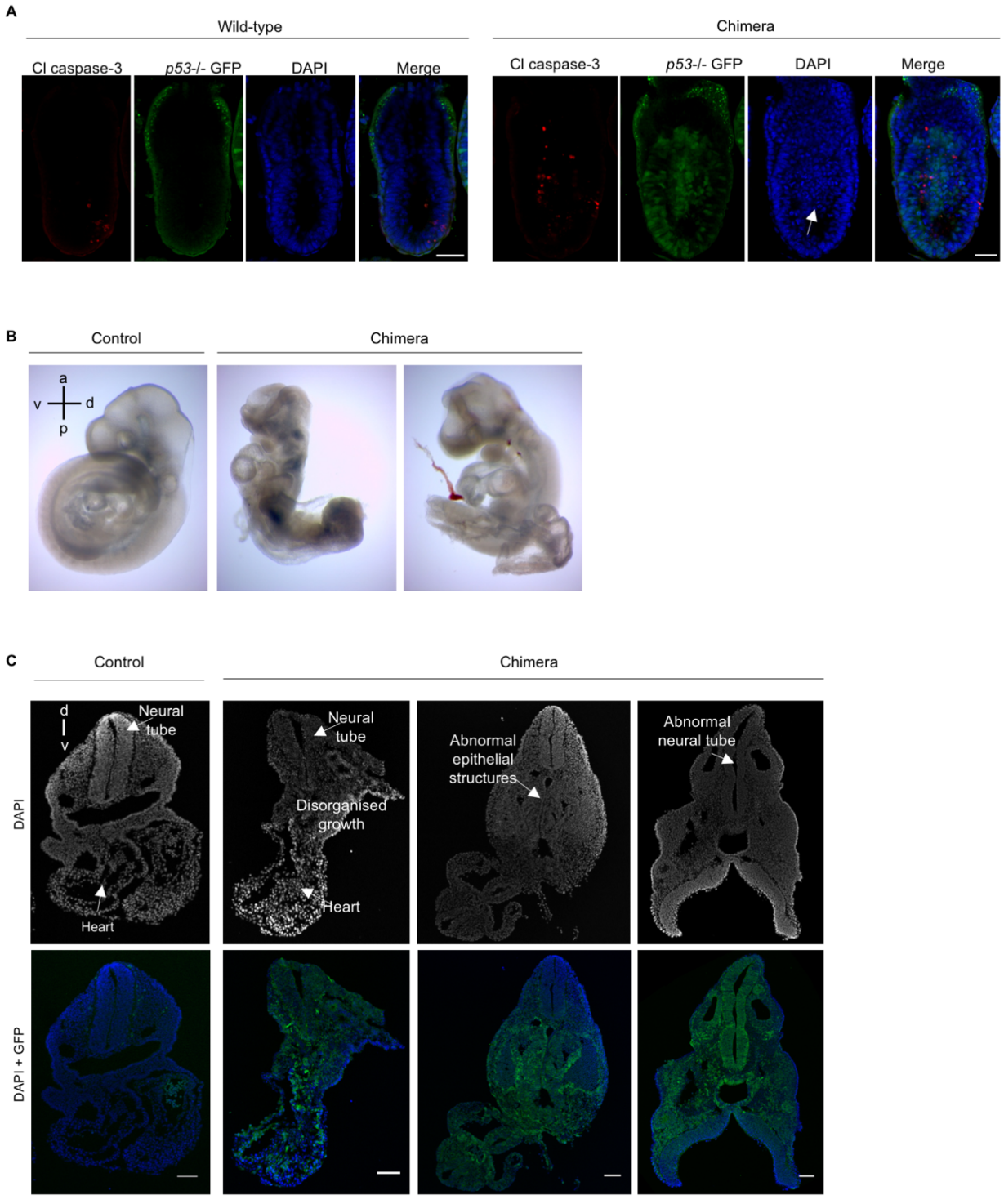


Figure 5.14: p53-mediated super-competition disrupts embryonic development.

A. *p53*$^{-/-}$ ESCs (GFP-labelled) were injected into wild-type blastocysts and cell death was analysed at E6.5 by staining for cl.caspase-3. B. *p53*$^{-/-}$ cells were injected into wild-type blastocysts and embryo morphology was analysed at E9.5. C. Sections were taken of *p53*$^{-/-}$/ wild-type chimeras at E9.5 and

stained for anti-GFP to detect *p53*$^{-/-}$ cells. Chimeras were generated by Jenny Nichols (University of Cambridge) and embryo dissection and analysis was performed by Aida di Gregorio (Imperial College London).

5.3. Discussion

In this chapter we aimed to identify upstream components of the pathway which leads to mTOR inhibition in defective cells during competition. Our results suggest that the observed reduction in mTOR activation is not a result of competition for a limited pool of nutrients in the environment, as we did not observe a rescue of mTOR activity following either addition of amino acids, insulin, or fresh media to competing cells. Similarly, knock-down of insulin-sensing pathway inhibitor PTEN failed to rescue pS6 levels. Instead, we mined our gene expression analysis for proteins which could act upstream of the mTOR pathway to repress its activity. We identified p53, which is elevated in loser cells and is required for mTOR pathway repression and loser cell elimination. The increased expression of two p53 target genes which encode mTOR-repressing proteins, *Redd1* and *Sesn1*, specifically in competing loser cells suggests that p53 could potentially undergo a change of activity during competition to repress mTOR. Finally, we found that heterogeneous p53 deletion leads to supercompetition which is sufficient to severely disrupt normal embryo morphogenesis.

We propose a model whereby loser cells are marked by an elevation of p53 levels, which could mediate competition by priming cells for competition-induced elimination or driving the expression of genes encoding proteins involved in fitness-communication (Fig. 5.14). In a competitive scenario, the mTOR pathway is repressed in loser cells. This could be a result of a change in p53 activity which directly drives the expression of mTOR-inhibitors *Redd1* and *Sesn1*. Alternatively, it could be a result of the activation of another 'competition-sensing' pathway which reduces mTOR levels in p53-marked loser cells. Together, our results indicate that p53 could be a sensor of reduced cell fitness and instigator cell competition, while mTOR acts as an effector of competition and driver of loser cell death.

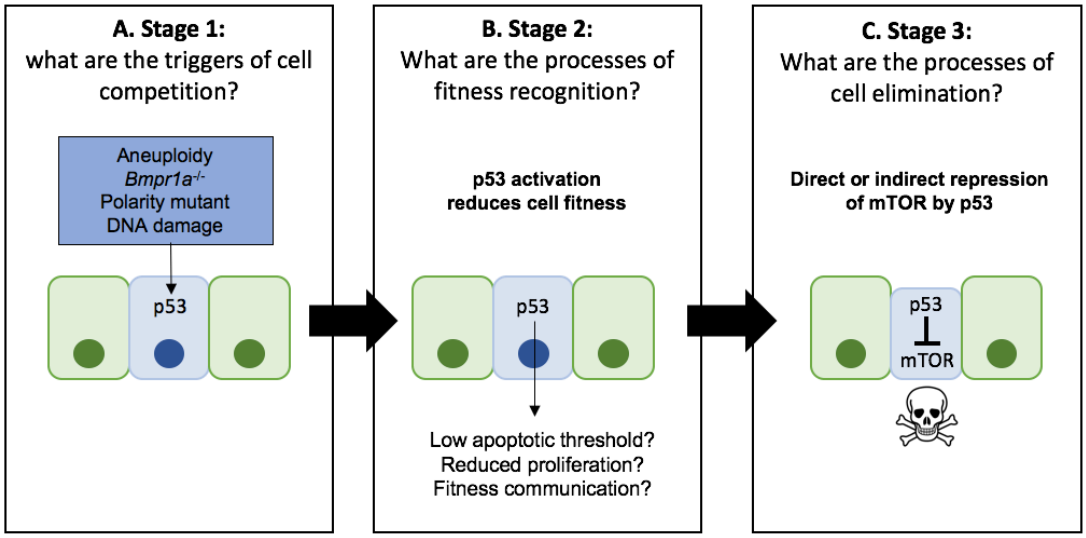


Figure 5.15: Elevated p53 marks loser cells and triggers their elimination when in a heterotypic environment.

A. We propose that a range of cell defects result in moderate stress and moderate p53 activation, including karyotypic abnormalities (Fig. 5.5C,D; Li et al., 2010), growth factor deprivation (Fig. 5.5A,B; Green et al., 2003), or disruption of cell polarity (Wagstaff et al., 2016). B. Elevated p53 mediates competition. This could be through passive means, such as by lowering the cell apoptotic threshold, reducing cell proliferation, or altering cellular metabolism; or through active means, such as by directing the expression of genes encoding proteins involved in fitness communication. C. In a heterotypic environment, the mTOR pathway becomes repressed and this results in cell death. Our data suggests that this could occur as a result of p53 directly changing activity to activate the transcription of genes which encode mTOR-repressing proteins. Alternatively, reduced *relative* growth/ apoptotic threshold/ glycolysis, or fitness communication described in (B) could directly lead to mTOR repression in loser cells.

5.3.1. Stage 1: Loser cells are marked by elevated levels of p53

Our study, in combination with others, suggests that p53 could act as a general marker of reduced cell fitness and therefore of potential loser cells. Both of our loser lines exhibit elevated p53 levels. This activation is presumably a result of mild stress: *Bmpr1a*$^{-/-}$ have disrupted BMP signalling, while tetraploid cells may have an activated DNA damage response. Accordingly, both growth factor deprivation (Green et al., 2013) and tetraploidy (Andreassen et al., 2001; Margolis et al., 2003) are known triggers of p53. Elevated p53 also marks loser cells in HSCs and MDCK cell competition models (Wagstaff et al., 2016; Bondar and Medzhitov, 2010). Together, these data demonstrate that several defects which render cells viable but less fit converge on p53, and the protein can act as a read-out of reduced cell fitness.

5.3.2. Stage 2: p53 activation in losers drives competition

As well as marking loser cells, our evidence when combined with others' suggests that the elevation of p53 in losers also has a functional role in driving competition. Two pieces of evidence support this. First, elevated p53 is required for elimination of defective cells (Fig. 5.7-9; Wagstaff et al., 2016; Bondar and Medzhitov, 2010). Second, the confrontation of cells with differential p53 activity is sufficient to drive competition: we have identified that wild-type cells with basal p53 levels are eliminated in the presence of *p53*$^{-/-}$ cells, while others have shown that the upregulation of p53 alone is sufficient to cause cells to become eliminated in wild-type mouse embryos (Zhang et al., 2017).

How could p53 differences mediate cell competition?

On the one hand, p53 differences could trigger cell competition through passive mechanisms. For example, p53 activation would reduce cell proliferation which could render cells sensitive to mechanical stress when surrounded by faster growing cells. In keeping with this, knockdown or deletion of p53 in loser cell lines increases their proliferation rate, indicating that elevated p53 slows proliferation (Fig. 5.7-9). Furthermore, the confrontation of cells with different proliferation rates has previously been shown experimentally and through mathematical modelling to result in mechanical compaction and elimination of the slower-growing cells (Shraiman et al, 2009; Levayer et al., 2016). This hypothesis does not explain p53-mediated super-competition however, as we did not observe differences in growth rates between wild-type and *p53*-null cells (Fig. 5.12).

Alternatively, elevated p53 could act to lower the apoptotic threshold of loser cells. Although it is feasible that this would be phenotypically silent in a low-stress homotypic environment, in a heterotypic environment, loser cells could incur higher levels of stress which triggers their apoptosis. This role for p53 has already been described in competing MDCK cells, where *Scribble*$^{-/-}$ cells have increased sensitivity to mechanical compaction by wild-type cells due to their increased levels of p53 (Wagstaff et al., 2016).

Another option is that p53 elevation alters the metabolic profile of cells which limits their fitness. p53 promotes the metabolic reprogramming of cells by promoting oxidative phosphorylation and inhibiting glycolysis (Bieging et al., 2014). During mouse ESC

differentiation to EpiSC, glycolysis is heavily upregulated at the expense of oxidative phosphorylation (Zhou et al., 2012). It is possible that by attenuating the efficiency of this switch, p53 activation reduces the ability of loser cells to thrive when surrounded by more glycolytic cells.

An alternative option is that p53 drives the expression of genes which encode proteins involved in fitness communication, thereby mediating 'active' cell competition. In *Drosophila*, "fitness fingerprints" have been purported to mediate cell competition through direct sensing of fitness state between cells. For example, polarity mutant cells in the imaginal wing disc express cell surface protein PTP10D which initiates a signalling pathway leading to cell death following 'recognition' of cell surface protein Sas on surrounding wild-type cells (Yamamoto et al., 2017). Similarly, competition driven by *Minute* heterozygosity and differential Myc levels is mediated by the expression of innate immunity receptors on losers in *Drosophila* (de la Cova et al., 2016). However, in these cases it is not clear what drives the expression of proteins which mark cells as losers in the first place. It is possible that p53 could act in a dose-dependent fashion to drive expression of cell-surface markers which communicate fitness status, or p53-activation level, to the surrounding cells. Alternatively, p53 could be driving the release of secreted factors which could communicate fitness. p53 has previously been described to drive non-cell-autonomous interactions through the regulation of secreted SASP proteins (Lujambio et al., 2013). It would be intriguing to investigate whether cells with relatively higher p53 levels could initiate apoptosis following the 'sensing' of surrounding cells with relatively lower levels of p53 activation using either cell-surface or secreted fitness codes.

5.3.3. Stage 3: How does p53 activation lead to mTOR inhibition in losers?

In chapter 4, our results demonstrated that mTOR inhibition in loser cells is an effector of cell competition. In this chapter we have identified that depletion of p53 in loser cells rescues mTOR pathway activation. Together, these findings reveal that p53 elevation leads to mTOR repression in outcompeted loser cells. Here I will discuss the possible mechanisms through which p53 elevation could lead to mTOR repression in loser cells.

First, it is possible that p53 directly acts to inhibit the mTOR pathway through expression of genes which encode proteins involved in mTOR repression. In keeping with this, we observed that the expression levels of p53-targets *Redd1* and *Sesn1* are elevated only in the co-culture condition, suggesting that p53 could undergo a change of activity specifically in co-culture.

This change in p53 activity in a competitive context could explain why loser cells in separate culture are viable and sustain high levels of mTOR activity, despite exhibiting elevated levels of p53. It is possible that reduced *relative* growth/ apoptotic threshold/ glycolysis or fitness recognition (outlined in *How could p53 elevation mediate cell competition?*) results in a change in p53 activity through post-translational modification.

Second, it is possible that p53 does not change activity, but that in a competitive environment mTOR is repressed through the reduced ability of cells to thrive in their environment, which is caused by p53 elevation. Possible causes are described above in *How could p53 elevation mediate cell competition?*

5.3.4. Comparison of our finding with other competition systems

The involvement of p53 in our system of competition bears similarity with other studies, as mentioned previously. However, there are some differences which we will discuss here.

First, our finding that deletion of *p53* alone is sufficient to turn cells into super-competitors differs from previous research into the role of p53 in cell competition: in MDCK and hematopoietic stem cells, deletion of *p53* triggers competition only if surrounding wild-type cells have elevated basal p53 levels as a result of Nutlin-3 treatment or DNA damage (Bondar and Medzhitov, 2010; Wagstaff et al., 2016). We propose that the differences between p53-mediated competition in our system and others could be due to the elevated basal levels of p53 in wild-type cells during differentiation due to its role in mesendoderm induction (Wang et al., 2017). In this case, during the early stages of mouse embryogenesis loss of p53 directly would create a disparity in p53 levels between the mutant and surrounding cells that is sufficient to trigger competitive interactions. Alternatively, the discrepancies could be a result of lower apoptotic threshold of differentiating ESCs (Heyer et al., 2000; Pernaute et al., 2014), or could arise as a result of different p53 target genes in our system.

Second, another difference between our study and others is that in our system p53 appears to change activity in a competitive environment to trigger loser cell apoptosis. This differs with MDCK cells, where p53 levels in loser cells are further increased to lethal thresholds upon culture with wild-type cells as a results of mechanical stress (Wagstaff et al, 2016). In HSCs, there is some evidence that p53 drives cell senescence in the presence of wild-type HSCs,

although it is unclear whether this senescent-type state is caused by cell competition rather than cell-autonomous activation (Bondar and Medzhitov, 2010). Therefore, the activity of p53 following competition may be cell type- or tissue-specific.

Finally, p53 has been described to have a very different role in *Drosophila* cell competition models. Specifically, in this system p53 is required in winner cells to eliminate losers (de la Cova et al., 2014). Furthermore, loser cell elimination occurs in the absence of p53 (Kale et al., 2015). As the stress-response pathway JNK is important for loser cell elimination in *Drosophila* (Moreno et al., 2002; Moreno et al., 2004; Tamori and Deng, 2013; Kolahger et al., 2015; Suijkerbuijk et al., 2016; Kucinski et al., 2017), it is possible that in flies, the stress response is mediated through JNK instead of p53. It will be interesting to determine if JNK has a role in loser cell elimination in early embryogenesis to test this.

Chapter 6: Concluding remarks

Cell competition is thought to act as a quality control mechanism and has primarily been studied in flies. More recently, the elimination of viable but suboptimal cells has been studied in the early mammalian embryo at the onset of gastrulation. However, the mechanisms responsible for either marking less fit cells or governing loser cell elimination have remained obscure. In this thesis, unbiased RNA expression profiling has been employed to identify two regulators of cell competition in the early mammalian embryo: the mTOR pathway and p53. While elevated levels of p53 mark less fit cells in separate culture and therefore act as an effector of reduced *intrinsic* cell fitness, downregulation of mTOR occurs in loser cells only in competition and therefore acts as an effector of reduced *relative* cell fitness. Differential levels of p53 and mTOR are required for cell competition to occur during embryogenesis, and are also sufficient to induce competition with deleterious consequences for morphogenesis. Having discussed the mechanisms of cell competition, here I will discuss the potential roles of the process in light of our findings, further implications of our results, and future work.

6.1. Roles of cell competition

6.1.1. Coupling of cell apoptosis to organismal need

Our data demonstrates that loser cells are marked by elevated levels of p53, and that these elevated levels are required for loser cell out-competition. p53 is emerging as a key player in cell competition in different systems. In polarity mutant MDCK cells, p53 is activated and is required for loser cell elimination in response to mechanical stress (Wagstaff et al., 2016), and in the mouse embryo, elevated levels of p53 induce competition (Zhang et al., 2017). Similarly, competition between wild-type hematopoietic stem/ progenitor cells and those subjected to DNA damaged selects for cells with lower p53 levels (Bondar and Medzhitov, 2010). Given that p53 responds to a huge number of cellular stresses, our results, together with those of others, suggest that p53 could act as a general sensor of reduced cell fitness across different tissues, and could function to remove non-lethally damaged cells only in a heterogeneous environment where there are surrounding healthier cells which can take their place (Fig. 6.1). p53-mediated cell competition therefore could provide organisms and tissues with a mechanism to remove sub-optimal cells only if they can afford to do so, and act to balance the benefit of removing a less-fit cell with the cost of losing a functional, albeit damaged, unit.

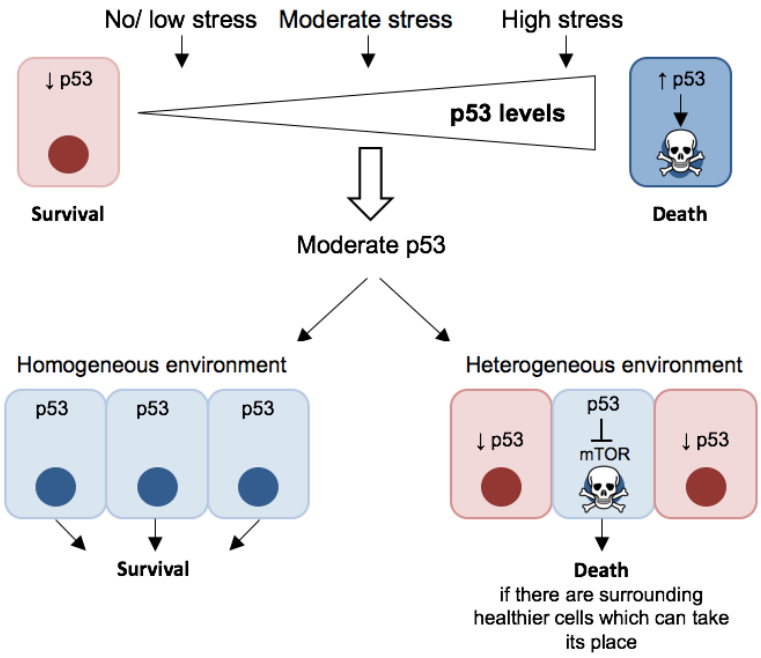


Figure 6.1: Role for cell competition in balancing cell elimination with organismal need.
Model for how different levels of p53 could modulate the type of cell death response. While high levels of activation leads to activation of the cell-intrinsic cell death pathways, moderate levels could be recognised by neighbouring cells as "less-fit" leading to their elimination.

6.1.2. Elimination of aneuploid cells during development

An increasing body of evidence suggests that aneuploid and polyploid cells are selected against both during development (Bolton et al., 2016; Greco et al., 2015; Sancho et al., 2013) and throughout life (Pfau et al., 2016). Furthermore, karyotypically abnormal cells are marked by elevated p53 levels (Li et al., 2010). Since the work in this thesis has revealed that differences in p53 are both sufficient and necessary for competition to occur, it is tempting to speculate that a possible endogenous role for cell competition is to eliminate karyotypically abnormal cells. We have shown that persistence of *Tsc2*$^{-/-}$ tetraploid cells in development leads to severe developmental abnormalities (Fig. 4.17). Although we need to perform further experiments to identify whether the observed phenotype is a result of mTOR hyperactivation or tetraploidy itself, the finding suggests that removal of karyotypically abnormal cells is necessary for proper embryo development. Later in life, aneuploidy is associated with aggressive tumour states which evade immune system detection (Davoli et al., 2017; Holland and Cleveland, 2009). Furthermore, deletion of p53 in tetraploid cells is sufficient to drive tumourigenesis (Fujiwara et al., 2005). Given the advantages of their removal, it is feasible that cell competition has evolved as a potent mechanism to remove cells with karyotypic abnormalities.

6.2. Understanding cell competition from a wider perspective

Together, the involvement of p53 and mTOR in competition has implications that go beyond the elimination of defective cells during development which could shed light on a diverse range of areas, from human stem cell culture mutations to cancer and organismal ageing.

6.2.1. Spread of mutations in stem cell cultures

Induced pluripotent stem cells (iPSCs) have held a huge amount of promise for regenerative medicine due to their ability to be generated from differentiated somatic cells and to re-differentiate into a range of cells for use in regenerative medicine or disease modelling (Singh et al., 2015). Their use circumvents the problems associated with donor organ availability and host rejection. However, numerous studies have indicated that cultured iPSCs are genetically and epigenetically unstable (summarised by Pera, 2011) and trials in Japan were halted recently due to the occurrence of both novel copy number variations and single nucleotide variations in cultured iPSCs derived from patients (Garber, 2015). Furthermore, it has been observed that dominant-negative p53 mutations are acquired in cultured hESCs and expand in number at the expense of wild-type cells (Merkle et al., 2017). In light of our data, it seems possible that the selective advantage gained by p53-mutant cells could be executed through cell competition. Since the propagation of such mutations would have dire consequences for therapies using induced pluripotent stem cells (iPSCs), understanding the nature of the mechanisms which promote cell competition will be paramount for optimising culture conditions for iPSCs, which otherwise have promising applications for regenerative medicine.

6.2.2. Supercompetition in cancer

The finding that deletion of *Tsc2* and *p53*, both known tumour suppressors, can trigger elimination of wild-type cells raises the possibility that the growth of these tumour types could be fuelled by cell competition. In particular, the epiblast overgrowths we see in wild-type/*p53*-null chimeras are reminiscent of unchecked proliferation. Cell competition has previously been shown to drive tumour growth and invasiveness (Ballesteros-Arias et al., 2014; Eichenlaub et al., 2016; Suijkerbuijk et al., 2016). It will be important to investigate whether cell competition promotes the aggressiveness of *Tsc2*-null or *p53*-null tumours as this finding could lead to the development of personalised therapies which target this particular driving force of

tumourigenesis, which has been sufficient to contain tumour growth in other models (Ballesteros-Arias et al., 2014; Eichenlaub et al., 2016; Suijkerbuijk et al., 2016).

6.2.3. Tissue-specific cancer drivers

Our study reveals that *p53*$^{-/-}$ ESCs trigger the elimination of wild-type cells. However, this differs with competition studies in hematopoietic stem cells and MDCK cells where supercompetition is not induced by *p53* deletion alone. Taken together, the findings suggest that mutations which induce supercompetition are tissue-specific. It is tempting to draw links between this and the tissue-specific nature of many cancer-driving mutations (Schaefer and Serrano, 2016; Schneider et al., 2017). The finding that many mutations cause cancers in some tissues but not others could potentially be in part explained by the different ability of mutations to induce competition in different tissues; some mutations may confer a fitness advantage in one tissue but not another. To investigate this hypothesis further it would be interesting to perform a screen in different cell types to identify genes which induce competition, and then compare supercompetitor mutations with cancer-driving genes and identify if there is a correlation between competition-driving mutations and cancer occurrence.

6.2.4. Persistence of less fit cells in ageing

The work in this thesis, and that of others before, demonstrates that p53 gradients formed between winner cells and loser cells have a crucial role in triggering cell competition (Bondar and Medzhitov, 2010; Wagstaff et al, 2016; Zhang et al, 2017). Notably, a sufficient gradient of p53 activation is required to trigger elimination of the higher-p53 cell (Bondar and Medzhitov, 2010; Wagstaff et al., 2016). When considered from the perspective of organismal ageing, this could have a number of important implications for cell competition dynamics.

The first implication draws from the expectation that general p53 levels increase with age as a result of increased tissue stress, DNA damage, and senescence (Hoeijmakers, 2009; López-Otín et al., 2013). As a result, damaged or aged tissues with elevated general levels of p53 will form less sizeable gradients of p53 between normal and defective cells. It is feasible therefore that the efficiency of defective cell elimination declines with age, leading to the persistence of defective cells and an overall decline in tissue function (Fig. 6.2). On the other hand, widespread elevation of p53 would result in the formation of more sizeable p53 activation gradients between normal cells and *p53*-null cells. This could therefore lead to an increased

selective advantage of *p53*-null cells in ages or damaged tissues. Intriguingly, this hypothesis is supported by the finding that *p53*-null intestinal stem cells gain a selective advantage in colitis-damaged intestines but not normal ones (Vermeulen et al., 2013). Potentially, this is due to an increased p53 gradient between $p53^{-/-}$ intestinal stem cells and surrounding colitis-damaged tissue which promotes their selective advantage. Furthermore, *p53*-null hematopoietic stem cells only gain a selective advantage in irradiated mice (Bondar and Medzhitov, 2010), while *p53*-null MDCK cells also only trigger supercompetition when surrounding cells are treated with p53 activator Nutlin-3a (Wagstaff et al., 2016). In summary, as several studies have demonstrated that a sufficient p53 activation gradient is required to trigger cell competition, the general increase in tissue p53 levels with age could lead to both a reduction in removal of defective cells, and also to an increase in the ability of *p53*-null cells to propagate.

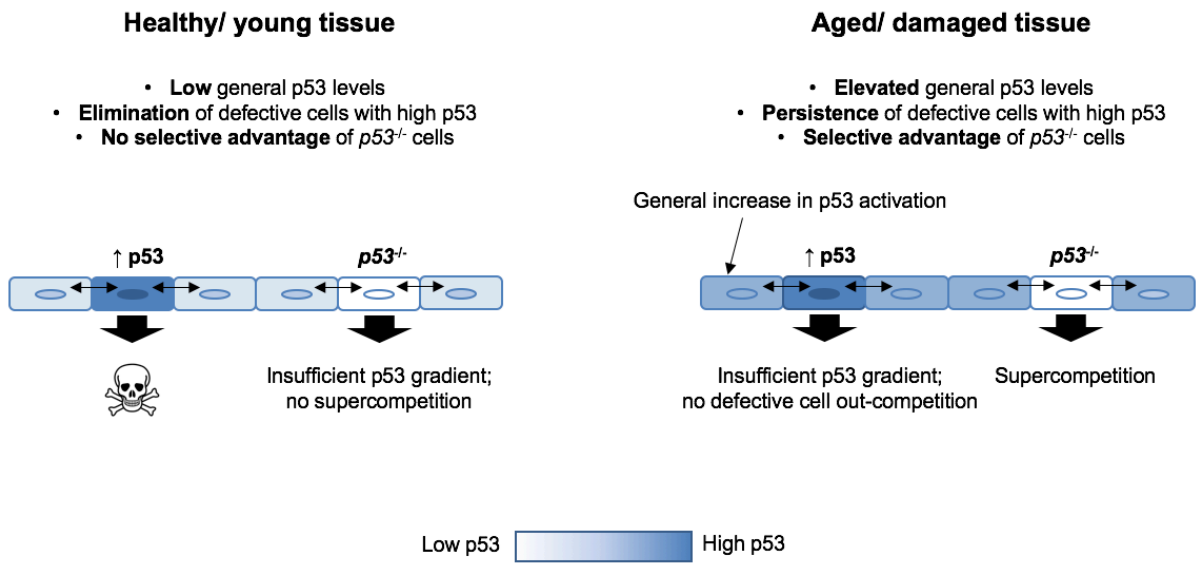


Figure 6.2: Model illustrating potential implications of p53-mediated cell competition in ageing and disease.

In healthy and/or young tissue, p53 levels would be expected to be low. This would create large gradient between defective and normal cells, triggering cell competition, but create a small gradient between $p53^{-/-}$ and normal cells, preventing supercompetition (as in Wagstaff et al., 2016; Bondar and Medzhitov et al., 2010). In aged and/or damaged tissue, damage and senescence would be expected to cause a general elevation in p53 levels. This would result in an insufficient gradient of p53 between defective an normal cells, leading to their persistence, and create a large gradient between $p53^{-/-}$ and normal cells, leading to supercompetition. These effects could contribute to the persistence of defective cells and selective advantage of 'super-fit' cells with age, leading to increased disease occurrence.

The second implication of our findings draws from evidence that p53 function declines with age (Feng et al., 2007b). Specifically, the ability of the protein to activate cell apoptosis is attenuated in aged mice due to its decreased stabilisation following stress (Feng et al., 2007b). This could result in a reduced ability of loser cells to trigger apoptosis following competitive interactions.

Taken together, it is feasible that cell competition dynamics could become altered during organismal ageing due to changes in both p53 activation and function. Understanding if this is the case may yield greater understanding into the rise of disease with age (Niccoli and Partridge, 2012). For example, the persistence of damaged cells could contribute to cancer formation or neurodegenerative disease. Supporting this, inhibition of cell competition by deletion of *Azot* in *Drosophila* leads to an increase in the number of neurodegenerative vacuoles and reduces the ability of tissues to recover from UV-induced stress (Merino et al., 2015). Conversely, the increased propensity of *p53*-null cells to colonise aged or damaged tissues could lead to an increase in field cancerization or an acceleration in tumour development. To test this speculation it would be intriguing to assess the efficiency of cell competition in aged tissues compared to normal ones and to investigate whether modulation of p53 levels affects the dynamics.

The work in this thesis suggests that a repression in the mTOR pathway is required for loser cell elimination. Therefore, it could be thought that repressing the mTOR pathway in all cells would reduce cell competition efficiency and have negative implications for healthy ageing. However, this is not reconciled by research in the ageing field: repression of the mTOR pathway through genetic or pharmaceutical means extends lifespan in numerous species (Niccoli and Partridge, 2012). These apparent contradictions could be aligned by considering that repression of the mTOR pathway may boost the elimination of less fit cells: by reducing mTOR pathway levels across all tissues, the less fit but living cells may be forced below a threshold of sufficient mTOR activation required for survival. This could permit the surival of only the healthiest cells with pre-existing high levels of mTOR activation. Supporting this, chronic rapamycin treatment has been found to increase the elimination of mutant cells in our system (George et al., unpublished).

6.3. Future work

Work in this thesis has shed further light on the mechanisms governing cell competition between differentiating ESCs; however, a number of questions still remain. For example, it will be important to determine how p53 activation mediates cell competition, and whether a change in p53 activity occurs during competition. Furthermore, it will be important to investigate the relevance of the uncovered mechanisms in cell competition *in vivo*. Here I will outline experiments which could be used to investigate how p53 regulates cell competition and which will allow us to confirm the involvement of p53 and mTOR in mediating cell competition *in vivo*. Furthermore, I will detail experiments which aim to understand physiological rates and roles of cell competition using p53 and mTOR as markers.

6.3.1. How does p53 activation mediate cell competition?

Our work in combination with others suggests that p53 mediates cell competition, although it is still not clear how this occurs. Future work would benefit from identifying the role of p53 in triggering cell competition. As previously discussed, elevation of p53 levels could render differentiating ESCs hypersensitive to mechanical stress as previously shown in MDCK cells (Wagstaff et al., 2016). To test this possibility, it will be important to investigate levels of caspase activation in wild-type and defective cell types with and without p53 following mechanical stress. This could be performed by seeding cells on stretched PDMS substrates and analysing death and p53 levels following the release of this substrate to induce compression of cells, as previously described (Wagstaff et al., 2016). This would allow us to identify if p53 expression increases the sensitivity of defective cells to mechanical stress.

We also postulated that p53 activation could reduce cell competitiveness by altering metabolism. p53 inhibits glycolysis through the transcription activation of *Tigar* (Bensaad et al., 2006) and repression of glucose transporters *GLUT1* and *GLUT4* (Schwartzenberg-Bar-Yoseph et al., 2004). In order to investigate whether p53 confers the loser state by affecting the metabolic profile of a cell, it will be interesting to identify if these genes are differentially activated between loser and winner cells. If so, this hypothesis could be functionally tested by depleting TIGAR in loser cells using CRISPR and identifying whether this rescues loser elimination.

In addition to the above, further study could benefit from an unbiased approach to understand p53 targets. Specifically, it could be productive to perform RNA-sequencing analysis of defective and wild-type cells with and without p53 depletion. This would allow us to identify changes in gene expression in loser cells and in 'rescued' loser cells (ie, those with depleted p53). Similarly, the analysis would allow us to compare gene expression in wild-type cells and supercompetitor *p53*$^{-/-}$ cells. In particular, it may be interesting to identify if any genes encoding cell-surface or secreted proteins are altering by manipulating p53, as these may be good contenders for proteins involved in cell-cell fitness sensing. Similarly, it may be interesting to use mass spectrometry to compare the proteins present in the media cultured with wild-type and *p53*$^{-/-}$ cells as another way to investigate possible secreted fitness-communicators.

6.3.2. Does p53 change activity during competition?

Although the upregulation of p53-targets *Redd1* and *Sesn1* in competition suggest that p53 undergoes a change of activity specifically in competing loser cells which acts to repress the mTOR pathway, it will be important to perform further experiments to confirm this hypothesis. First, it will be necessary to confirm that the expression of these genes is driven by p53. This could be achieved by ensuring that mRNA levels of *Redd1* and *Sesn1* are no longer increased in p53-depleted competing loser cells. Second, it will be necessary to confirm that the increased expression of these genes is important for mTOR repression. This could be achieved by depleting cells of *Redd1* and *Sesn1* by CRISPR and ensuring that mTOR repression and elimination of defective cells no longer occurs. It will also be interesting to understand whether p53 undergoes a change of activity in wild-type cells undergoing competition in the presence of *p53*$^{-/-}$ cells. Although we did not see evidence for mTOR repression in p53-mediated supercompetition, hyperactivation of mTOR rescued wild-type elimination, suggesting that a reduction in pS6 may be observed at other time-points.

6.3.3. Do p53 and mTOR drive cell competition *in vivo*?

The majority of our work has been performed using an *in vitro* system which is designed to mimic cell competition during gastrulation. It will next be important to validate our results to assess whether p53 and mTOR have important roles in orchestrating cell competition *in vivo*. For example, while we identified that tetraploid cells lose pS6 staining in wild-type embryos, it will be important to demonstrate that these cells are also marked by elevated p53 *in vivo*. Furthermore, while we have shown that hyperactivation of the mTOR pathway is sufficient to rescue 4n elimination in wild-type chimeras, it will also be important to demonstrate that p53

deletion in these cells rescues both their elimination and downregulation of pS6. Interestingly, it has already been demonstrated that deletion of p53 allows tetraploid cells to contribute to adult diploid mice (Horii et al, 2015), suggesting that our *in vitro* findings can be validated *in vivo*.

6.3.4. Using p53 and mTOR as markers to track endogenous cell competition

A significant outstanding question in cell competition research is: what are the physiological roles of the process? Most cell competition research has been performed using non-physiological models which artificially induce cell competition. As a result, the endogenous rates and roles of the process remain unresolved. Using either elevated p53 or low mTOR pathway activation as markers, it will be possible to identify the endogenous rates and roles of cell competition during early mouse embryogenesis.

Identifying endogenous rates of cell competition

We can identify rates of cell competition by examining loss of mTOR pathway activation in gastrulating embryos normally and in the absence of cell death (Fig. 6.3a). In our *in vitro* cell culture system, inhibition of cell death using caspase inhibitors inhibits competition and leads to an accumulation of low-pS6 cells which would otherwise be outcompeted (Fig. 4.6A-C). By comparing the number of low pS6 cells in embryos treated with and without caspase inhibitors at E6.5, we will be able to determine the flux of cell competition at this stage in the embryo.

Identifying endogenous roles of cell competition

We can also use low-pS6 as a marker of competition to identify the endogenous roles of the process during gastrulation (Fig. 6.3b). Since we have postulated that cell competition could function to remove aneuploid cells at gastrulation, it will be interesting to identify if cells with low pS6 in caspase-treated embryos show signs of aneuploidy using fluorescence *in situ* hybridisation. To gain a more comprehensive and unbiased idea of differences between winner and loser cells in the developing embryo, it would be interesting to sort cells in caspase-inhibitor treated embryos based on pS6 levels and profile gene expression, pathway activation status, and membrane protein content to identify differences between these low- and high-pS6 cells, which represent loser and winner cells respectively. This could be carried out by treating embryos with caspase inhibitors, staining for pS6, sorting cells based on pS6 levels and then performing RNA sequencing (Hrvatin et al., 2014), phospho-proteomic analysis and mass

spectrometry of membrane proteins (Laganowsky et al., 2013). With these analyses we would be able to identify if out-competed cells share signatures which could either mark them as losers, allow communication of fitness status, or cause cells to be eliminated.

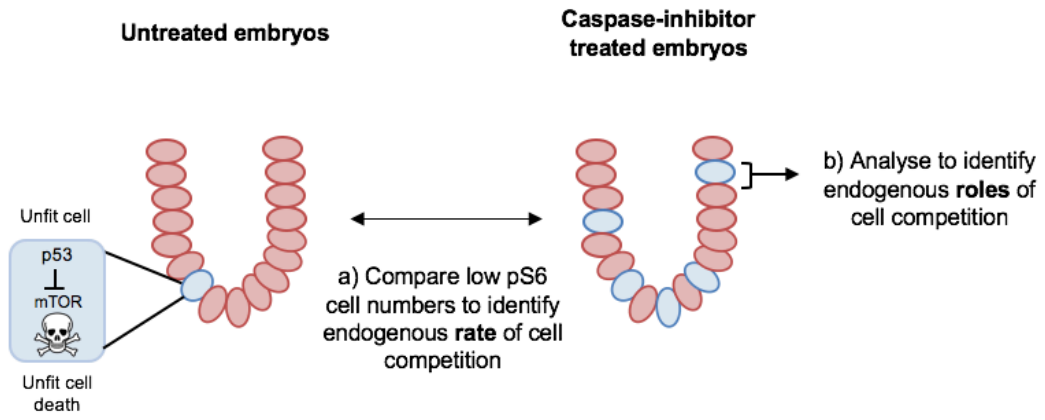


Figure 6.3: Assay to study rate and role of cell competition during early mouse embryogenesis. Culturing gastrulating embryos in the presence of caspase inhibitors will prevent the elimination of cells. We can use this as a tool to study the flux of cell competition (by comparing the proportion of low pS6 cells in normal and caspase-inhibitor treated embryos), and to study the role of competition (by assessing profiling aneuploidy levels/ gene expression/ signal transduction pathway activity in low pS6 cells).

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. Developmental Biology *172*, 218-229.

Akeno, N., Miller, A.L., Ma, X., and Wikenheiser-Brokamp, K.A. (2015). p53 suppresses carcinoma progression by inhibiting mTOR pathway activation. Oncogene *34*, 589-599.

Alcolea, M.P., Greulich, P., Wabik, A., Frede, J., Simons, B.D., and Jones, P.H. (2014). Differentiation imbalance in single oesophageal progenitor cells causes clonal immortalization and field change. Nat Cell Biol *16*, 612-619.

Altschuler, S.J., and Wu, L.F. (2010). Cellular Heterogeneity: Do Differences Make a Difference? Cell *141*, 559-563.

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

Andreassen, P.R., Lohez, O.D., Lacroix, F.B., and Margolis, R.L. (2001). Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. Mol Biol Cell *12*, 1315-1328.

Baar, M.P., Brandt, R.M.C., Putavet, D.A., Klein, J.D.D., Derks, K.W.J., Bourgeois, B.R.M., Stryeck, S., Rijksen, Y., van Willigenburg, H., Feijtel, D.A.*, et al.* (2017). Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. Cell *169*, 132-147.e116.

Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., A. Saltness, R., Jeganathan, K.B., Verzosa, G.C., Pezeshki, A.*, et al.* (2016). Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. Nature *530*, 184-189.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16(Ink4a)-positive senescent cells delays ageing-associated disorders. Nature *479*, 232-236.

Baker, N.E. (2011). Cell competition. Current Biology *21*, R11-R15.

Ballesteros-Arias, L., Saavedra, V., and Morata, G. (2014). Cell competition may function either as tumour-suppressing or as tumour-stimulating factor in Drosophila. Oncogene *33*, 4377-4384.

Bar-Peled, L., Chantranupong, L., Cherniack, A.D., Chen, W.W., Ottina, K.A., Grabiner, B.C., Spear, E.D., Carter, S.L., Meyerson, M., and Sabatini, D.M. (2013). A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science *340*, 1100-1106.

Beddington, R.S.P., and Robertson, E.J. (1999). Axis Development and Early Asymmetry in Mammals. Cell *96*, 195-209.

Bedzhov, I., and Zernicka-Goetz, M. (2014). Self-organizing properties of mouse pluripotent cells initiate morphogenesis upon implantation. Cell *156*, 1032-1044.

Ben-Sahra, I., Howell, J.J., Asara, J.M., and Manning, B.D. (2013). Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. Science *339*, 1323-1328.

Ben-Sahra, I., Hoxhaj, G., Ricoult, S.J.H., Asara, J.M., and Manning, B.D. (2016). mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science *351*, 728-733.

Bensaad, K., Tsuruta, A., Selak, M.A., Vidal, M.N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K.H. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell *126*, 107-120.

Berkers, C.R., Maddocks, O.D., Cheung, E.C., Mor, I., and Vousden, K.H. (2013). Metabolic regulation by p53 family members. Cell Metab *18*, 617-633.

Betschinger, J., Nichols, J., Dietmann, S., Corrin, P.D., Paddison, P.J., and Smith, A. (2013). Exit from pluripotency is gated by intracellular redistribution of the bHLH transcription factor Tfe3. Cell *153*, 335-347.

Beugnet, A., Tee, A.R., Taylor, P.M., and Proud, C.G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. Biochemical Journal *372*, 555-566.

Bieging, K.T., Mello, S.S., and Attardi, L.D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer *14*, 359-370.

Bilanges, B., Argonza-Barrett, R., Kolesnichenko, M., Skinner, C., Nair, M., Chen, M., and Stokoe, D. (2007). Tuberous sclerosis complex proteins 1 and 2 control serum-dependent translation in a TOP-dependent and -independent manner. Mol Cell Biol *27*, 5746-5764.

Bolton, H., Graham, S.J.L., 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. Nature Communications *7*, 11165.

Bondar, T., and Medzhitov, R. (2010). p53-mediated hematopoietic stem and progenitor cell competition. Cell Stem Cell *6*, 309-322.

Borek, C., and Sachs, L. (1966). The difference in contact inhibition of cell replication between normal cells and cells transformed by different carcinogens. Proceedings of the National Academy of Sciences of the United States of America *56*, 1705-1711.

Braakhuis, B.J.M., Tabor, M.P., Kummer, J.A., Leemans, C.R., and Brakenhoff, R.H. (2003). A Genetic Explanation of Slaughter's Concept of Field Cancerization. Cancer Research *63*, 1727.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., and Kaelin, W.G. (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes & Development *18*, 2893-2904.

Brumby, A.M., and Richardson, H.E. (2003). *scribble* mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*. The EMBO Journal *22*, 5769.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr., and Abraham, R.T. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science *277*, 99-101.

Budanov, A.V., and Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell *134*, 451-460.

Burke, R., and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. Development *122*, 2261.

Busch, K., Klapproth, K., Barile, M., Flossdorf, M., Holland-Letz, T., Schlenner, S.M., Reth, M., Hofer, T., and Rodewald, H.-R. (2015). Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. Nature *518*, 542-546.

Böhni, 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.

Cam, M., Bid, H.K., Xiao, L., Zambetti, G.P., Houghton, P.J., and Cam, H. (2014). p53/TAp63 and AKT regulate mammalian target of rapamycin complex 1 (mTORC1) signaling through two independent parallel pathways in the presence of DNA damage. J Biol Chem *289*, 4083-4094.

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.W.*, et al.* (2015). Metabolic competition in the tumor microenvironment is a driver of cancer progression. Cell *162*, 1229-1241.

Chantranupong, L., Scaria, S.M., Saxton, R.A., Gygi, M.P., Shen, K., Wyant, G.A., Wang, T., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2016). The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. Cell *165*, 153-164.

Chantranupong, L., Wolfson, R.L., Orozco, J.M., Saxton, R.A., Scaria, S.M., Bar-Peled, L., Spooner, E., Isasa, M., Gygi, S.P., and Sabatini, D.M. (2014). The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. Cell Rep *9*, 1-8.

Chauvin, C., Koka, V., Nouschi, A., Mieulet, V., Hoareau-Aveilla, C., Dreazen, A., Cagnard, N., Carpentier, W., Kiss, T., Meyuhas, O.*, et al.* (2014). Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. Oncogene *33*, 474-483.

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. Proceedings of the National Academy of Sciences *109*, 484-489.

Cherepkova, M.Y., Sineva, G.S., and Pospelov, V.A. (2016). Leukemia inhibitory factor (LIF) withdrawal activates mTOR signaling pathway in mouse embryonic stem cells through the MEK/ERK/TSC2 pathway. Cell Death Dis *7*, e2050.

Cherry, S.R., Biniszkiewicz, D., van Parijs, L., Baltimore, D., and Jaenisch, R. (2000). Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol Cell Biol *20*, 7419-7426.

Claveria, C., Giovinazzo, G., Sierra, R., and Torres, M. (2013). Myc-driven endogenous cell competition in the early mammalian embryo. Nature *500*, 39-44.

Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C., and Piccolo, S. (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. Cell *113*, 301-314.

Darwin, C. (1859). On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life (London: J. Murray).

Davoli, T., Uno, H., Wooten, E.C., and Elledge, S.J. (2017). Tumor aneuploidy correlates with markers of immune evasion and with reduced response to immunotherapy. Science *355*.

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.

Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., and Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA-[beta]gal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protocols *4*, 1798-1806.

Dejosez, M., Ura, H., Brandt, V.L., and Zwaka, T.P. (2013). Safeguards for Cell Cooperation in Mouse Embryogenesis Shown by Genome-Wide Cheater Screen. Science *341*, 1511.

Demaria, M., Ohtani, N., Youssef, S.A., Rodier, F., Toussaint, W., Mitchell, J.R., Laberge, R.-M., Vijg, J., Van Steeg, H., Dollé, M.E.T.*, et al.* (2014). An Essential Role for Senescent Cells in Optimal Wound Healing through Secretion of PDGF-AA. Developmental cell *31*, 722-733.

Demetriades, C., Plescher, M., and Teleman, A.A. (2016). Lysosomal recruitment of TSC2 is a universal response to cellular stress. Nat Commun *7*, 10662.

Deppmann, C.D., Mihalas, S., Sharma, N., Lonze, B.E., Niebur, E., and Ginty, D.D. (2008). A Model for Neuronal Competition During Development. Science *320*, 369.

DeYoung, M.P., Horak, P., Sofer, A., Sgroi, D., and Ellisen, L.W. (2008). Hypoxia regulates TSC1/2–mTOR signaling and tumor suppression through REDD1-mediated 14–3–3 shuttling. Genes & Development *22*, 239-251.

Dhanasekaran, D.N., and Reddy, E.P. (2008). JNK Signaling in Apoptosis. Oncogene *27*, 6245-6251.

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.

Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., and Manning, B.D. (2012). TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. Mol Cell *47*, 535-546.

Ding, M., Bruick, R.K., and Yu, Y. (2016). Secreted IGFBP5 mediates mTORC1-dependent feedback inhibition of IGF-1 signalling. Nat Cell Biol *18*, 319-327.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S.*, et al.* (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell *39*, 171-183.

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., Chang, S., Gumper, I., Snitkin, H., Wolfson, R.L., Kirak, O., Sabatini, D.D., and Sabatini, D.M. (2013). Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. Nature *493*, 679-683.

Eichenlaub, T., Cohen, S.M., and Herranz, H. (2016). Cell Competition Drives the Formation of Metastatic Tumors in a Drosophila Model of Epithelial Tumor Formation. Curr Biol *26*, 419-427.

Eisenhoffer, G.T., Loftus, P.D., Yoshigi, M., Otsuna, H., Chien, C.-B., Morcos, P.A., and Rosenblatt, J. (2012). Crowding induces live cell extrusion to maintain homeostatic cell numbers in epithelia. Nature *484*, 546-549.

Ellisen, L.W., Ramsayer, K.D., Johannessen, C.M., Yang, A., Beppu, H., Minda, K., Oliner, J.D., McKeon, F., and Haber, D.A. (2002). REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. Mol Cell *10*, 995-1005.

Fellmann, C., Hoffmann, T., Sridhar, V., Hopfgartner, B., Muhar, M., Roth, M., Lai, D.Y., Barbosa, I.A., Kwon, J.S., Guan, Y.*, et al.* (2013). An optimized microRNA backbone for effective single-copy RNAi. Cell Rep *5*, 1704-1713.

Feng, Z., Hu, W., de Stanchina, E., Teresky, A.K., Jin, S., Lowe, S., and Levine, A.J. (2007a). The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res *67*, 3043-3053.

Feng, Z., Hu, W., Teresky, A.K., Hernando, E., Cordon-Cardo, C., and Levine, A.J. (2007b). Declining p53 function in the aging process: A possible mechanism for the increased tumor incidence in older populations. Proceedings of the National Academy of Sciences *104*, 16633-16638.

Feng, Z., and Levine, A.J. (2010). The regulation of energy metabolism and the IGF-1/mTOR pathways by the p53 protein. Trends Cell Biol *20*, 427-434.

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. Proceedings of the National Academy of Sciences of the United States of America *102*, 8204-8209.

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. Nature neuroscience *16*, 1373-1382.

Fodde, R. (2002). The APC gene in colorectal cancer. European Journal of Cancer *38*, 867-871.

French, J.B., Jones, S.A., Deng, H., Pedley, A.M., Kim, D., Chan, C.Y., Hu, H., Pugh, R.J., Zhao, H., Zhang, Y.*, et al.* (2016). Spatial colocalization and functional link of purinosomes with mitochondria. Science *351*, 733-737.

Fu, C., and Ess, K.C. (2013). Conditional and domain-specific inactivation of the Tsc2 gene in neural progenitor cells. Genesis *51*, 284-292.

Fujiwara, T., Bandi, M., Nitta, M., Ivanova, E.V., Bronson, R.T., and Pellman, D. (2005). Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature *437*, 1043-1047.

Gangloff, Y.-G., Mueller, M., Dann, S.G., Svoboda, P., Sticker, M., Spetz, J.-F., Um, S.H., Brown, E.J., Cereghini, S., Thomas, G.*, et al.* (2004). Disruption of the Mouse mTOR Gene Leads to Early Postimplantation Lethality and Prohibits Embryonic Stem Cell Development. Molecular and Cellular Biology *24*, 9508-9516.

Garber, K. (2015). RIKEN suspends first clinical trial involving induced pluripotent stem cells. Nat Biotech *33*, 890-891.

Gatenby, R.A., and Gawlinski, E.T. (1996). A reaction-diffusion model of cancer invasion. Cancer Res *56*, 5745-5753.

Gaussin, V., Van de Putte, T., Mishina, Y., Hanks, M.C., Zwijsen, A., Huylebroeck, D., Behringer, R.R., and Schneider, M.D. (2002). Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. Proceedings of the National Academy of Sciences of the United States of America *99*, 2878-2883.

Gibson, M.C., and Perrimon, N. (2005). Extrusion and Death of DPP/BMP-Compromised Epithelial Cells in the Developing *Drosophila* Wing. Science *307*, 1785.

Gingras, A.C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem *68*, 913-963.

Godar, S., Ince, T.A., Bell, G.W., Feldser, D., Donaher, J.L., Bergh, J., Liu, A., Miu, K., Watnick, R.S., Reinhardt, F.*, et al.* (2008). Growth-inhibitory and tumor- suppressive functions of p53 depend on its repression of CD44 expression. Cell *134*, 62-73.

Gottlieb, R.A., Giesing, H.A., Zhu, J.Y., Engler, R.L., and Babior, B.M. (1995). Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H(+)-ATPase. Proc Natl Acad Sci U S A *92*, 5965-5968.

Greco, E., Minasi, M.G., and Fiorentino, F. (2015). Healthy Babies after Intrauterine Transfer of Mosaic Aneuploid Blastocysts. New England Journal of Medicine *373*, 2089-2090.

Green, B.D., Jabbour, A.M., Sandow, J.J., Riffkin, C.D., Masouras, D., Daunt, C.P., Salmanidis, M., Brumatti, G., Hemmings, B.A., Guthridge, M.A.*, et al.* (2013). Akt1 is the principal Akt isoform regulating apoptosis in limiting cytokine concentrations. Cell Death Differ *20*, 1341-1349.

Green, D.R., and Kroemer, G. (2009). Cytoplasmic functions of the tumour suppressor p53. Nature *458*, 1127-1130.

Hafezi, Y., Bosch, J.A., and Hariharan, I.K. (2012). Differences in levels of the transmembrane protein Crumbs can influence cell survival at clonal boundaries. Developmental Biology *368*, 358-369.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

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. Developmental Biology *152*, 233-241.

Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C.J., Raguz, S., Acosta, J.C., Innes, A.J., Banito, A.*, et al.* (2015). mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. Nat Cell Biol *17*, 1205-1217.

Heyer, B.S., MacAuley, A., Behrendtsen, O., and Werb, Z. (2000). Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. Genes & Development *14*, 2072-2084.

Hoeijmakers, J.H.J. (2009). DNA Damage, Aging, and Cancer. New England Journal of Medicine *361*, 1475-1485.

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.

Holland, A.J., and Cleveland, D.W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol *10*, 478-487.

Hrvatin, S., Deng, F., O'Donnell, C.W., Gifford, D.K., and Melton, D.A. (2014). MARIS: Method for Analyzing RNA following Intracellular Sorting. PLOS ONE *9*, e89459.

Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., Bonham, M.J.*, et al.* (2012). The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature *485*, 55-61.

Hu, W., Feng, Z., Teresky, A.K., and Levine, A.J. (2007). p53 regulates maternal reproduction through LIF. Nature *450*, 721-724.

Huang, T., and Rivera-Pérez, J.A. (2014). Senescence-associated β-galactosidase activity marks the visceral endoderm of mouse embryos but is not indicative of senescence. genesis *52*, 300-308.

Inoki, K., Li, Y., Xu, T., and Guan, K.-L. (2003a). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes & Development *17*, 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Inoki, K., Ouyang, H., Zhu, T., Lindvall, C., Wang, Y., Zhang, X., Yang, Q., Bennett, C., Harada, Y., Stankunas, K.*, et al.* (2006). TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell *126*, 955-968.

Inoki, K., Zhu, T., and Guan, K.L. (2003b). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

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 (New York, NY) *326*, 153-156.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat Signaling Mediates Regeneration and Homeostasis in the Drosophila Midgut. Cell *137*, 1343-1355.

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.

Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., and Gallant, P. (1999). Drosophila myc Regulates Cellular Growth during Development. Cell *98*, 779-790.

Johnston, L.A., and Sanders, A.L. (2003). Wingless promotes cell survival but constrains growth during Drosophila wing development. Nat Cell Biol *5*, 827-833.

Jung, J., Genau, H.M., and Behrends, C. (2015). Amino Acid-Dependent mTORC1 Regulation by the Lysosomal Membrane Protein SLC38A9. Mol Cell Biol *35*, 2479-2494.

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. Journal of Cell Science *123*, 171.

Kajita, M., Sugimura, K., Ohoka, A., Burden, J., Suganuma, H., Ikegawa, M., Shimada, T., Kitamura, T., Shindoh, M., Ishikawa, S.*, et al.* (2014). Filamin acts as a key regulator in epithelial defence against transformed cells. Nature Communications *5*, 4428.

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.

Kalender, A., Selvaraj, A., Kim, S.Y., Gulati, P., Brule, S., Viollet, B., Kemp, B.E., Bardeesy, N., Dennis, P., Schlager, J.J.*, et al.* (2010). Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. Cell Metab *11*, 390-401.

Kaufman, M.H. (1991). Histochemical identification of primordial germ cells and differentiation of the gonads in homozygous tetraploid mouse embryos. Journal of Anatomy *179*, 169-181.

Kaufman, M.H. (1992). Postcranial morphological features of homozygous tetraploid mouse embryos. Journal of Anatomy *180*, 521-534.

Kaur, J., and Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. Nat Rev Mol Cell Biol *16*, 461-472.

Kawamura, N., Sun-Wada, G.-H., Aoyama, M., Harada, A., Takasuga, S., Sasaki, T., and Wada, Y. (2012). Delivery of endosomes to lysosomes via microautophagy in the visceral endoderm of mouse embryos. Nat Commun *3*, 1071.

Kenzelmann Broz, D., Spano Mello, S., Bieging, K.T., Jiang, D., Dusek, R.L., Brady, C.A., Sidow, A., and Attardi, L.D. (2013). Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. Genes Dev *27*, 1016-1031.

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol *13*, 132-141.

Kimball, S.R., Dang Do, A.N., Kutzler, L., Cavener, D.R., and Jefferson, L.S. (2008). Rapid Turnover of the mTOR Complex 1 (mTORC1) Repressor REDD1 and Activation of mTORC1 Signaling Following Inhibition of Protein Synthesis. The Journal of biological chemistry *283*, 3465-3475.

Kirschner, K., Samarajiwa, S.A., Cairns, J.M., Menon, S., Perez-Mancera, P.A., Tomimatsu, K., Bermejo-Rodriguez, C., Ito, Y., Chandra, T., Narita, M.*, et al.* (2015). Phenotype specific analyses reveal distinct regulatory mechanism for chronically activated p53. PLoS Genet *11*, e1005053.

Kojima, Y., Tam, O.H., and Tam, P.P.L. (2014). Timing of developmental events in the early mouse embryo. Seminars in Cell & Developmental Biology *34*, 65-75.

Kolahgar, G., Suijkerbuijk, Saskia J.E., Kucinski, I., Poirier, Enzo Z., Mansour, S., Simons, Benjamin D., and Piddini, E. (2015). Cell Competition Modifies Adult Stem Cell and Tissue Population Dynamics in a JAK-STAT-Dependent Manner. Developmental Cell *34*, 297-309.

Kon, S., Ishibashi, K., Katoh, H., Kitamoto, S., Shirai, T., Tanaka, S., Kajita, M., Ishikawa, S., Yamauchi, H., Yako, Y.*, et al.* (2017). Cell competition with normal epithelial cells promotes apical extrusion of transformed cells through metabolic changes. Nat Cell Biol *19*, 530-541.

Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. Cell *134*, 657-667.

Kruiswijk, F., Labuschagne, C.F., and Vousden, K.H. (2015). p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat Rev Mol Cell Biol *16*, 393-405.

Kruse, J.P., and Gu, W. (2009). Modes of p53 regulation. Cell *137*, 609-622.

Kucinski, I., Dinan, M., Kolahgar, G., and Piddini, E. (2017). Chronic activation of JNK JAK/STAT and oxidative stress signalling causes the loser cell status. Nat Commun *8*, 136.

Laberge, R.M., Sun, Y., Orjalo, A.V., Patil, C.K., Freund, A., Zhou, L., Curran, S.C., Davalos, A.R., Wilson-Edell, K.A., Liu, S.*, et al.* (2015). MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. Nat Cell Biol *17*, 1049-1061.

Laganowsky, A., Reading, E., Hopper, J.T.S., and Robinson, C.V. (2013). Mass Spectrometry of Intact Membrane Protein Complexes. Nature protocols *8*, 639-651.

Laker, C., Meyer, J., Schopen, A., Friel, J., Heberlein, C., Ostertag, W., and Stocking, C. (1998). Host cis-Mediated Extinction of a Retrovirus Permissive for Expression in Embryonal Stem Cells during Differentiation. Journal of Virology *72*, 339-348.

Laurent, A., and Blasi, F. (2015). Differential DNA damage signalling and apoptotic threshold correlate with mouse epiblast-specific hypersensitivity to radiation. Development *142*, 3675.

Lee, B.Y., Han, J.A., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., and Hwang, E.S. (2006). Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell *5*, 187-195.

Lee, D.F., Kuo, H.P., Chen, C.T., Hsu, J.M., Chou, C.K., Wei, Y., Sun, H.L., Li, L.Y., Ping, B., Huang, W.C.*, et al.* (2007). IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. Cell *130*, 440-455.

Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., and Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development *122*, 3195-3205.

Leung, C.T., and Brugge, J.S. (2012). Outgrowth of Single Oncogene-expressing Cells from Suppressive Epithelial Environments. Nature *482*, 410-413.

Levayer, R., Dupont, C., and Moreno, E. (2016). Tissue Crowding Induces Caspase-Dependent Competition for Space. Current Biology *26*, 670-677.

Li, M., Fang, X., Baker, D.J., Guo, L., Gao, X., Wei, Z., Han, S., van Deursen, J.M., and Zhang, P. (2010). The ATM–p53 pathway suppresses aneuploidy-induced tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 14188-14193.

Li, W., and Baker, N.E. (2007). Engulfment Is Required for Cell Competition. Cell *129*, 1215-1225.

Lin, T., Chao, C., Saito, S., Mazur, S.J., Murphy, M.E., Appella, E., and Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol *7*, 165-171.

Liu, Y., Elf, S.E., Miyata, Y., Sashida, G., Huang, G., Di Giandomenico, S., Lee, J.M., Deblasio, A., Menendez, S., Antipin, J.*, et al.* (2009). p53 regulates hematopoietic stem cell quiescence. Cell Stem Cell *4*, 37-48.

Lolo, F.-N., Casas-Tintó, S., and Moreno, E. (2012). Cell Competition Time Line: Winners Kill Losers, which Are Extruded and Engulfed by Hemocytes. Cell Reports *2*, 526-539.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005). Rheb binds and regulates the mTOR kinase. Curr Biol *15*, 702-713.

Lujambio, A., Akkari, L., Simon, J., Grace, D., Tschaharganeh, D.F., Bolden, J.E., Zhao, Z., Thapar, V., Joyce, J.A., Krizhanovsky, V.*, et al.* (2013). Non-cell-autonomous tumor suppression by p53. Cell *153*, 449-460.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The Hallmarks of Aging. Cell *153*, 1194-1217.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P.P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell *121*, 179-193.

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. Journal of Cell Science *128*, 790.

Mammoto, T., and Ingber, D.E. (2010). Mechanical control of tissue and organ development. Development (Cambridge, England) *137*, 1407-1420.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

Margolis, R.L., Lohez, O.D., and Andreassen, P.R. (2003). G1 tetraploidy checkpoint and the suppression of tumorigenesis. J Cell Biochem *88*, 673-683.

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.

Martina, J.A., Chen, Y., Gucek, M., and Puertollano, R. (2012). MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. Autophagy *8*, 903-914.

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.

Martín, F.A., Herrera, S.C., and Morata, G. (2009). Cell competition, growth and size control in the *Drosophila* wing imaginal disc. Development *136*, 3747.

Marygold, S.J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G.H., Harrison, P.M., Yu, Z., Kenmochi, N., Kaufman, T.C.*, et al.* (2007). The ribosomal protein genes and Minute loci of Drosophila melanogaster. Genome Biology *8*, R216-R216.

Meletis, K., Wirta, V., Hede, S.M., Nister, M., Lundeberg, J., and Frisen, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. Development *133*, 363-369.

Menon, S., Dibble, C.C., Talbott, G., Hoxhaj, G., Valvezan, A.J., Takahashi, H., Cantley, L.C., and Manning, B.D. (2014). Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. Cell *156*, 771-785.

Menéndez, J., Pérez-Garijo, A., Calleja, M., and Morata, G. (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proceedings of the National Academy of Sciences *107*, 14651-14656.

Merino, Marisa M., Rhiner, C., Lopez-Gay, Jesus M., Buechel, D., Hauert, B., and Moreno, E. (2015). Elimination of Unfit Cells Maintains Tissue Health and Prolongs Lifespan. Cell *160*, 461-476.

Merino, Marisa M., Rhiner, C., Portela, M., and Moreno, E. (2013). "Fitness Fingerprints" Mediate Physiological Culling of Unwanted Neurons in Drosophila. Current Biology *23*, 1300-1309.

Merkle, F.T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C., Kashin, S., Mekhoubad, S., Ilic, D., Charlton, M.*, et al.* (2017). Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. Nature.

Meyer, S.N., Amoyel, M., Bergantiños, 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 (New York, NY) *346*, 1258236-1258236.

Milán, M., Pérez, L., and Cohen, S.M. (2002). Short-Range Cell Interactions and Cell Survival in the Drosophila Wing. Developmental Cell *2*, 797-805.

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 & Development *9*, 3027-3037.

Moellering, R.E., Black, K.C., Krishnamurty, C., Baggett, B.K., Stafford, P., Rain, M., Gatenby, R.A., and Gillies, R.J. (2008). Acid treatment of melanoma cells selects for invasive phenotypes. Clin Exp Metastasis *25*, 411-425.

Morata, G., and Ripoll, P. (1975). Minutes: Mutants of Drosophila autonomously affecting cell division rate. Developmental Biology *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.

Mungamuri, S.K., Yang, X., Thor, A.D., and Somasundaram, K. (2006). Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res *66*, 4715-4724.

Munoz-Espin, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol *15*, 482-496.

Murakami, M., Ichisaka, T., Maeda, M., Oshiro, N., Hara, K., Edenhofer, F., Kiyama, H., Yonezawa, K., and Yamanaka, S. (2004). mTOR Is Essential for Growth and Proliferation in Early Mouse Embryos and Embryonic Stem Cells. Molecular and Cellular Biology *24*, 6710-6718.

Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S., Rodríguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M.*, et al.* (2013). Programmed Cell Senescence during Mammalian Embryonic Development. Cell *155*, 1104-1118.

Narita, M., Young, A.R., Arakawa, S., Samarajiwa, S.A., Nakashima, T., Yoshida, S., Hong, S., Berry, L.S., Reichelt, S., Ferreira, M.*, et al.* (2011). Spatial coupling of mTOR and autophagy augments secretory phenotypes. Science *332*, 966-970.

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. Developmental Cell *19*, 507-520.

Niccoli, T., and Partridge, L. (2012). Ageing as a Risk Factor for Disease. Current Biology *22*, R741-R752.

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. Journal of Cell Science *125*, 59.

O'Brien, Lucy E., Soliman, Sarah S., Li, X., and Bilder, D. (2011). Altered Modes of Stem Cell Division Drive Adaptive Intestinal Growth. Cell *147*, 603-614.

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.

Ohsawa, S., Sugimura, K., Takino, K., Xu, T., Miyawaki, A., and Igaki, T. (2011). Elimination of Oncogenic Neighbors by JNK-Mediated Engulfment in Drosophila. Developmental Cell *20*, 315-328.

Oliver, E.R., Saunders, T.L., Tarlé, S.A., and Glaser, T. (2004). Ribosomal protein L24 defect in Belly spot and tail (Bst), a mouse Minute. Development *131*, 3907.

Olivier, M., Hollstein, M., and Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol *2*, a001008.

Onda, H., Lueck, A., Marks, P.W., Warren, H.B., and Kwiatkowski, D.J. (1999). Tsc2(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. J Clin Invest *104*, 687-695.

Oppenheim, R.W. (1985). Naturally occurring cell death during neural development. Trends in Neurosciences *8*, 487-493.

Paca, A., Séguin, C.A., Clements, M., Ryczko, M., Rossant, J., Rodriguez, T.A., and Kunath, T. (2012). BMP signaling induces visceral endoderm differentiation of XEN cells and parietal endoderm. Developmental Biology *361*, 90-102.

Panić, L., Tamarut, S., Sticker-Jantscheff, M., Barkić, M., Solter, D., Uzelac, M., Grabušić, K., and Volarević, S. (2006). Ribosomal Protein S6 Gene Haploinsufficiency Is Associated with Activation of a p53-Dependent Checkpoint during Gastrulation. Molecular and Cellular Biology *26*, 8880-8891.

Park, H.J., Lyons, J.C., Ohtsubo, T., and Song, C.W. (1999). Acidic environment causes apoptosis by increasing caspase activity. Br J Cancer *80*, 1892-1897.

Pearce, L.R., Alton, G.R., Richter, D.T., Kath, J.C., Lingardo, L., Chapman, J., Hwang, C., and Alessi, D.R. (2010). Characterization of PF-4708671, a novel and highly specific inhibitor of p70 ribosomal S6 kinase (S6K1). Biochem J *431*, 245-255.

Pena-Llopis, S., Vega-Rubin-de-Celis, S., Schwartz, J.C., Wolff, N.C., Tran, T.A., Zou, L., Xie, X.J., Corey, D.R., and Brugarolas, J. (2011). Regulation of TFEB and V-ATPases by mTORC1. Embo J *30*, 3242-3258.

Peng, M., Yin, N., and Li, M.O. (2017). SZT2 dictates GATOR control of mTORC1 signalling. Nature *543*, 433-437.

Penzo-Méndez, 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.

Pera, M.F. (2011). Stem cells: The dark side of induced pluripotency. Nature *471*, 46-47.

Perea-Gomez, A., Vella, F.D.J., 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. Developmental Cell *3*, 745-756.

Pernaute, B., Spruce, T., Smith, K.M., Sanchez-Nieto, J.M., Manzanares, M., Cobb, B., and Rodriguez, T.A. (2014). MicroRNAs control the apoptotic threshold in primed pluripotent stem cells through regulation of BIM. Genes Dev *28*, 1873-1878.

Peterson, T.R., Sengupta, S.S., Harris, T.E., Carmack, A.E., Kang, S.A., Balderas, E., Guertin, D.A., Madden, K.L., Carpenter, A.E., Finck, B.N.*, et al.* (2011). mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. Cell *146*, 408-420.

Pfau, S.J., Silberman, R.E., Knouse, K.A., and Amon, A. (2016). Aneuploidy impairs hematopoietic stem cell fitness and is selected against in regenerating tissues in vivo. Genes & Development *30*, 1395-1408.

Piette, J., Neel, H., and Marechal, V. (1997). Mdm2: keeping p53 under control. Oncogene *15*, 1001-1010.

Porstmann, T., Santos, C.R., Griffiths, B., Cully, M., Wu, M., Leevers, S., Griffiths, J.R., Chung, Y.L., and Schulze, A. (2008). SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. Cell Metab *8*, 224-236.

Prober, D.A., and Edgar, B.A. (2000). Ras1 Promotes Cellular Growth in the Drosophila Wing. Cell *100*, 435-446.

Qi, X., Li, T.G., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y., Zhao, G.Q. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. PNAS *101*, 6027-6032.

Raff, M.C. (1992). Social controls on cell survival and cell death. Nature *356*, 397-400.

Rebsamen, M., Pochini, L., Stasyk, T., de Araujo, M.E., Galluccio, M., Kandasamy, R.K., Snijder, B., Fauster, A., Rudashevskaya, E.L., Bruckner, M.*, et al.* (2015). SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. Nature *519*, 477-481.

Rhiner, C., Díaz, B., Portela, M., Poyatos, J.F., Fernández-Ruiz, I., López-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.

Rhiner, C., López-Gay, J.M., Soldini, D., Casas-Tinto, S., Martín, F.A., Lombardía, L., and Moreno, E. (2010). Flower Forms an Extracellular Code that Reveals the Fitness of a Cell to its Neighbors in Drosophila. Developmental Cell *18*, 985-998.

Ritsma, L., Ellenbroek, S.I.J., Zomer, A., Snippert, H.J., de Sauvage, F.J., Simons, B.D., Clevers, H., and van Rheenen, J. (2014). Intestinal crypt homeostasis revealed at single-stem-cell level by in vivo live imaging. Nature *507*, 362-365.

Rivera-Perez, J.A., and Hadjantonakis, A.K. (2014). The Dynamics of Morphogenesis in the Early Mouse Embryo. Cold Spring Harb Perspect Biol *7*.

Roczniak-Ferguson, A., Petit, C.S., Froehlich, F., Qian, S., Ky, J., Angarola, B., Walther, T.C., and Ferguson, S.M. (2012). The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. Sci Signal *5*, ra42.

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.

Rousseau, A., and Bertolotti, A. (2016). An evolutionarily conserved pathway controls proteasome homeostasis. Nature *536*, 184-189.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A *101*, 13489-13494.

Roux, W. (1881). Der Kampf der Theile im Organismus [The struggle of the parts in the organism] (Leipzig: W. Engelmann).

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev *19*, 2199-2211.

Ryoo, H.D., Gorenc, T., and Steller, H. (2004). Apoptotic Cells Can Induce Compensatory Cell Proliferation through the JNK and the Wingless Signaling Pathways. Developmental Cell *7*, 491-501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell *141*, 290-303.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell *25*, 903-915.

Sancho, M., Di-Gregorio, A., George, N., Pozzi, S., Sánchez, Juan M., Pernaute, B., and Rodríguez, Tristan A. (2013). Competitive Interactions Eliminate Unfit Embryonic Stem Cells at the Onset of Differentiation. Developmental cell *26*, 19-30.

Sansregret, L., and Swanton, C. (2017). The Role of Aneuploidy in Cancer Evolution. Cold Spring Harbor Perspectives in Medicine *7*.

Santorelli, L.A., Thompson, C.R.L., Villegas, E., Svetz, J., Dinh, C., Parikh, A., Sucgang, R., Kuspa, A., Strassmann, J.E., Queller, D.C., *et al.* (2008). Facultative cheater mutants reveal the genetic complexity of cooperation in social amoebae. Nature *451*, 1107-1110.

Sasai, N., Yakura, R., Kamiya, D., Nakazawa, Y., and Sasai, Y. (2008). Ectodermal factor restricts mesoderm differentiation by inhibiting p53. Cell *133*, 878-890.

Saxton, R.A., Chantranupong, L., Knockenhauer, K.E., Schwartz, T.U., and Sabatini, D.M. (2016). Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. Nature *536*, 229-233.

Saxton, R.A., and Sabatini, D.M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. Cell *168*, 960-976.

Schaefer, M.H., and Serrano, L. (2016). Cell type-specific properties and environment shape tissue specificity of cancer genes. Scientific Reports *6*, 20707.

Schalm, S.S., and Blenis, J. (2002). Identification of a Conserved Motif Required for mTOR Signaling. Current Biology *12*, 632-639.

Schalm, S.S., Tee, A.R., and Blenis, J. (2005). Characterization of a Conserved C-terminal Motif (RSPRR) in Ribosomal Protein S6 Kinase 1 Required for Its Mammalian Target of Rapamycin-dependent Regulation. Journal of Biological Chemistry *280*, 11101-11106.

Schlesinger, S., Lee, A.H., Wang, G.Z., Green, L., and Goff, S.P. (2013). Proviral silencing in embryonic cells is regulated by Yin Yang 1. Cell Rep *4*, 50-58.

Schmid, P., Lorenz, A., Hameister, H., and Montenarh, M. (1991). Expression of p53 during mouse embryogenesis. Development *113*, 857-865.

Schneider, G., Schmidt-Supprian, M., Rad, R., and Saur, D. (2017). Tissue-specific tumorigenesis: context matters. Nat Rev Cancer *17*, 239-253.

Schwartzenberg-Bar-Yoseph, F., Armoni, M., and Karnieli, E. (2004). The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res *64*, 2627-2633.

Senoo-Matsuda, N., and Johnston, L.A. (2007). Soluble factors mediate competitive and cooperative interactions between cells expressing different levels of Drosophila Myc. Proceedings of the National Academy of Sciences *104*, 18543-18548.

Sermeus, A., and Michiels, C. (2011). Reciprocal influence of the p53 and the hypoxic pathways. Cell Death Dis *2*, e164.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras Provokes Premature Cell Senescence Associated with Accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Settembre, C., Zoncu, R., Medina, D.L., Vetrini, F., Erdin, S., Huynh, T., Ferron, M., Karsenty, G., Vellard, M.C., Facchinetti, V.*, et al.* (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. Embo j *31*, 1095-1108.

Shigeta, M., Ohtsuka, S., Nishikawa-Torikai, S., Yamane, M., Fujii, S., Murakami, K., and Niwa, H. (2013). Maintenance of pluripotency in mouse ES cells without Trp53. Sci Rep *3*, 2944.

Shraiman, B.I. (2005). Mechanical feedback as a possible regulator of tissue growth. Proceedings of the National Academy of Sciences of the United States of America *102*, 3318-3323.

Simpson, P. (1979). Parameters of cell competition in the compartments of the wing disc of Drosophila. Developmental Biology *69*, 182-193.

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. Frontiers in Cell and Developmental Biology *3*, 2.

Siudeja, K., Nassari, S., Gervais, L., Skorski, P., Lameiras, S., Stolfa, D., Zande, M., Bernard, V., Frio, T.R., Bardin, A.J. (2015). Frequent Somatic Mutation in Adult Intestinal Stem Cells Drives Neoplasia and Genetic Mosaicism during Aging. Cell Stem Cell *17*, 663-674

Slaughter, D.P., Southwick, H.W., and Smejkal, W. (1953). “Field cancerization” in oral stratified squamous epithelium. Clinical implications of multicentric origin. Cancer *6*, 963-968.

Snippert, H.J., Schepers, A.G., van Es, J.H., Simons, B.D., and Clevers, H. (2013). Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. EMBO reports *15*, 62.

Snow, M.H.L. (1975). Embryonic development of tetraploid mice during the second half of gestation. Journal of Embryology and Experimental Morphology *34*, 707.

Solter, D., Škreb, N., and Damjanov, I. (1971). Cell cycle analysis in the mouse egg-cylinder. Experimental Cell Research *64*, 331-334.

Stern, C.D. (2008). Aptly Named Visceral Endoderm. Developmental Cell *15*, 493-494.

Stine, R.R., and Matunis, E.L. (2013). Stem cell competition: finding balance in the niche. Trends in Cell Biology *23*, 357-364.

Stoker, M. (1964). Regulation of growth and orientation in hamster cells transformed by polyoma virus. Virology *24*, 165-174.

Stoker, M.G.P., Shearer, M., and Neill, C. (1966). Growth Inhibition of Polyoma-Transformed Cells by Contact with Static Normal Fibroblasts. Journal of Cell Science *1*, 297.

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J.*, et al.* (2013). Senescence Is a Developmental Mechanism that Contributes to Embryonic Growth and Patterning. Cell *155*, 1119-1130.

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, J., Ramos, A., Chapman, B., Johnnidis, J.B., Le, L., Ho, Y.-J., Klein, A., Hofmann, O., and Camargo, F.D. (2014a). Clonal dynamics of native haematopoiesis. Nature *514*, 322-327.

Sun, Q., Luo, T., Ren, Y., Florey, O., Shirasawa, S., Sasazuki, T., Robinson, D.N., and Overholtzer, M. (2014b). Competition between human cells by entosis. Cell Res *24*, 1299-1310.

Swindle, C.S., Kim, H.G., and Klug, C.A. (2004). Mutation of CpGs in the Murine Stem Cell Virus Retroviral Vector Long Terminal Repeat Represses Silencing in Embryonic Stem Cells. Journal of Biological Chemistry *279*, 34-41.

Tamori, Y., and Deng, W.-M. (2013). Tissue repair through cell competition and compensatory cellular hypertrophy in postmitotic epithelia. Developmental cell *25*, 350-363.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol *13*, 1259-1268.

Teich, N.M., Weiss, R.A., Martin, G.R., and Lowy, D.R. (1977). Virus infection of murine teratocarcinoma stem cell lines. Cell *12*, 973-982.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature *485*, 109-113.

Tyler, D.M., Li, W., Zhuo, N., Pellock, B., and Baker, N.E. (2007). Genes Affecting Cell Competition in Drosophila. Genetics *175*, 643.

Vadysirisack, D.D., Baenke, F., Ory, B., Lei, K., and Ellisen, L.W. (2011). Feedback control of p53 translation by REDD1 and mTORC1 limits the p53-dependent DNA damage response. Mol Cell Biol *31*, 4356-4365.

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. Human Reproduction Update *17*, 620-627.

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.

Vermeulen, L., Morrissey, E., van der Heijden, M., Nicholson, A.M., Sottoriva, A., Buczacki, S., Kemp, R., Tavaré, S., and Winton, D.J. (2013). Defining Stem Cell Dynamics in Models of Intestinal Tumor Initiation. Science *342*, 995.

Villa del Campo, C., Clavería, C., Sierra, R., and Torres, M. (2014). Cell Competition Promotes Phenotypically Silent Cardiomyocyte Replacement in the Mammalian Heart. Cell Reports *8*, 1741-1751.

Villunger, A., Michalak, E.M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036-1038.

Vincent, J.-P., Kolahgar, G., Gagliardi, M., and Piddini, E. (2011). Steep Differences in Wingless Signaling Trigger Myc-Independent Competitive Cell Interactions. Developmental Cell *21*, 366-374.

Volarevic, S., Stewart, M.J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S.C., and Thomas, G. (2000). Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. Science *288*, 2045-2047.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

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.

Wang, Q., Zou, Y., Nowotschin, S., Kim, S.Y., Li, Q.V., Soh, C.L., Su, J., Zhang, C., Shu, W., Xi, Q.*, et al.* (2017). The p53 Family Coordinates Wnt and Nodal Inputs in Mesendodermal Differentiation of Embryonic Stem Cells. Cell Stem Cell *20*, 70-86.

Wang, S., Tsun, Z.Y., Wolfson, R.L., Shen, K., Wyant, G.A., Plovanich, M.E., Yuan, E.D., Jones, T.D., Chantranupong, L., Comb, W.*, et al.* (2015). Metabolism. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. Science *347*, 188-194.

Williams, A.C., Collard, T.J., and Paraskeva, C. (1999). An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. Oncogene *18*, 3199-3204.

Wolf, D., and Goff, S.P. (2007). TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell *131*, 46-57.

Wolf, D., and Goff, S.P. (2009). Embryonic stem cells use ZFP809 to silence retroviral DNAs. Nature *458*, 1201-1204.

Wolfson, R.L., Chantranupong, L., Saxton, R.A., Shen, K., Scaria, S.M., Cantor, J.R., and Sabatini, D.M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. Science *351*, 43-48.

Wolfson, R.L., Chantranupong, L., Wyant, G.A., Gu, X., Orozco, J.M., Shen, K., Condon, K.J., Petri, S., Kedir, J., Scaria, S.M*., et al.* (2017). KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. Nature *543*, 438-442.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656-660.

Yamamoto, M., Ohsawa, S., Kunimasa, K., and Igaki, T. (2017). The ligand Sas and its receptor PTP10D drive tumour-suppressive cell competition. Nature *542*, 246-250.

Zhang, G., Xie, Y., Zhou, Y., Xiang, C., Chen, L., Zhang, C., Hou, X., Chen, J., Zong, H., and Liu, G. (2017). p53 pathway is involved in cell competition during mouse embryogenesis. Proceedings of the National Academy of Sciences *114*, 498-503.

Zhao, J., Zhai, B., Gygi, S.P., and Goldberg, A.L. (2015). mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. Proc Natl Acad Sci U S A *112*, 15790-15797.

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). HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. The EMBO Journal *31*, 2103-2116.

Ziosi, M., Baena-López, L.A., Grifoni, D., Froldi, F., Pession, A., Garoia, F., Trotta, V., Bellosta, P., Cavicchi, S., and Pession, A. (2010). dMyc Functions Downstream of Yorkie to Promote the Supercompetitive Behavior of Hippo Pathway Mutant Cells. PLOS Genetics *6*, e1001140.

Appendix

Table 1: shRNA/ CRISPR target sequences

shRNA or CRISPR gene target	Sequence
Tsc2 #1 shRNA	AGCTGTTACCTTGACGAAT
Tsc2 # 2 shRNA	TGGACTACAAGTGCAACCT
Pten shRNA	CAGCTAAAGGTGAAGATAT
p53 CRISPR	GCAGACTTTTCGCCACAGCG
TSC2 CRISPR 2.1	CAAAGATTCCGGCTTGA
TSC2 CRISPR 2.2	TGGGATTGGGAACATCG
TSC2 CRISPR 3.1	TTCGGATGCGATTGTTG
TSC2 CRISPR 3.2	AATCGCATCCGAATGAT
TSC2 CRISPR 36.1	TGACAAGAAACGGCACC
TSC2 CRISPR 36.2	GACAAGAAACGGCACCT

Table 2: Primers for cloning

Primer	Sequence
5'-pGIPZ-XhoI	TACAATACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG
3'-pGIPZ-EcoRI	TTAGATGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCA

Table 3: Antibodies

Antibody	Application
Phospho-rpS6$^{S240/244}$ (Cell Signalling, #5264)	IF, WB, flow cytometry
Phospho-rpS6$^{S235/236}$ (Cell Signalling #4858	WB
Total rpS6 (Cell Signalling #2217)	WB
Phospho-p70 S6K^{T389} (Cell Signalling 9205)	WB
p53 (Cell Signalling #2524)	WB
Phospho-4E-BP1$^{T37/46}$ (Cell Signalling #2855)	IF, WB
Total 4E-BP1 (Cell Signalling #9644)	WB
Phospho-p90 RSK^{S380} (Cell Signalling #11989)	WB

α-tubulin (Cell Signalling #2144)	WB
β-actin (Cell Signalling #4970)	WB
Cl. caspase-3 asp175 (Cell Signalling #9664)	WB, flow cytometry
Cl. PARP (Cell Signalling #9544)	WB
Phospho-AKT^{T473} (Cell Signalling #4060)	WB
TSC2 (Cell Signalling #4308)	WB
PTEN (Cell Signalling #)	WB
p53 (CM5, Novocastra)	IF
CD31 (BD Biosciences, MEC13.3)	Flow cytometry
λH2AX (Milipore, 05-636)	IF

Table 4: Primer sequences used for qPCR

Gene	**Primer fw (5'→3')**	**Primer rv (5'→3')**
mTRP53	CACGTACTCTCCTCCCCTCAAT	AACTGCACAGGGCACGTCTT
TSC2	TGCCGCAGCATCAGTGTATC	TGCCAGGAGGAACTCTCCC
PTEN	TGGATTCGACTTAGACTTGACCT	GCGGTGTCATAATGTCTCTCAG
AMPKβ1	AGGCCCAAGATCCTCATGGA	GGGGGCTTTATCATTCGCTTC
REDD1	CAAGGCAAGAGCTGCCATAG	CCGGTACTTAGCGTCAGGG
SESN1	CGGACCAAGCAGGTTCATCC	TGATGTTATCCAGACGACCCAAA
SESN2	TCCGAGTGCCATTCCGAGAT	TCCGGGTGTAGACCCATCAC
p21	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT
Nanog	CTTACAAGGGTCTGCTACTGAGATGC	TGCTTCCTGGCAAGGACCTT
Esrrb	AACCGAATGTCGTCCGAAGAC	GTGGCTGAGGGCATCAATG
Rex1	CGAGTGGCAGTTTCTTCTTGG	GACTCACTTCCAGGGGGCAC
Klf4	ACCTATACCAAGAGTTCTCATC	TCTGGCACTGAAAGGGCCGG
β-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA