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The role of Myc in the ground state of pluripotency

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1. Summary

Pluripotency in the early embryo is defined as the capacity of a single cell to generate all lineages of the adult organism. *In vivo* this property is possessed only transiently by the cells of the epiblast, but it can be indefinitely "captured" *in vitro* by deriving embryonic stem (ES) cells from the inner cell mass of the blastocyst. A second property that characterizes ES cells is self-renewal, the capacity to generate more stem cells. Self-renewal requires the coordination of cell proliferation and cell-fate choice (Orford and Scadden, 2008). Originally, mouse ES cells were expanded on mitotically inactivated fibroblasts, in a culture medium containing fetal bovine serum and the leukemia inhibitory factor (LIF). Under these conditions ES cells are metastable, since they show heterogeneity both in morphology and expression of the core pluripotency factors Oct4 and Nanog due to differentiation signals coming from the serum components (Nichols and Smith, 2009). In serum + LIF medium, ES cells depend on the transcription factor and proto-oncogene Myc for pluripotency and self-renewal (Smith et al., 2010). Myc proteins have been shown to repress the expression of the primitive endoderm master regulator Gata6, and control the cell cycle by regulating the mir-17-92 miRNA cluster. The laboratory of Austin Smith has recently shown that the culture in the absence of serum but in the presence of two inhibitors (2i + LIF) of the Erk and glycogen synthase kinase-3 (GSK3) pathways is sufficient to stabilize ES cells in a more naïve, so-called ground state of pluripotency, which more closely resembles the status of the inner cell mass of the blastocyst (Ying et al., 2008). Since different culture environments impose distinctive transcriptional and epigenetic properties on mouse ES cells (Marks et al., 2012), we re-evaluated the expression and requirement of the Myc proteins in the naïve state of pluripotency.

Making use of ES cells expressing a Myc-GFP reporter from the endogenous *c-myc* locus, we found that c-Myc protein expression is significantly lower in naïve ES cells compared to cells cultured in serum + LIF. When both c- and N-myc genes are conditionally deleted (Myc dKO) using the Cre-loxP system, naïve mouse ES cells undergo cell cycle arrest. Although this type of cell cycle behavior with a decreased

rate of cell division is often associated with differentiation, Myc dKO ES cells form smaller but undifferentiated colonies. Most surprisingly, Myc dKO ES cells maintain the expression of the core stem cell factor network including Oct4, Nanog and Sox2 both at the RNA and the protein level. To determine the molecular properties of Myc dKO ES cells, we performed RNA-seq analysis 24 and 96 hours after Cre induction. Our results show that Myc is not exclusively regulating the cell cycle machinery but is directly and indirectly involved in multiple aspects of ES cell metabolism. In the absence of both Myc proteins, expression of genes controlling and executing ribosomal biogenesis as well as protein and DNA synthesis is strongly down-regulated, leading to a state of "metabolic dormancy".

The state of "metabolic dormancy" of ES cells *in vitro* resembled the state of embryonic diapause *in vivo*. Diapause is a poorly understood phenomenon of reversible arrest of embryonic development prior to implantation. In mice, facultative diapause occurs to delay implantation of newly formed embryos when the mother is feeding a previous litter. When we compared our data to reported global gene expression profiling of diapause embryos (Hamatani et al., 2004), we observed that dKO Myc ES cells possess surprising similarity with the dormant blastocyst, characterized by reduced DNA synthesis and cell division, low metabolic activity and activation of the insulin pathway (Given, 1988; Hamatani et al., 2004).

These data suggest that, in the absence of differentiation cues, c-Myc and N-Myc control the cell cycle of ES cells and the transcriptional networks responsible for the entire metabolism and biosynthesis pathways while the pluripotency network is controlled by other means. Loss of Myc activity has dramatic consequences on the metabolic status of ES cells promoting their entry into a state of likely reversible dormancy closely resembling arrested diapause embryos.

2. Zusammenfassung

Pluripotenz ist definiert als die Fähigkeit früher embryonaler Zellen jede Zelle des Organismus bilden zu können. *In vivo* besteht diese Eigenschaft im Epiblast nur vorübergehend, während sie *in vitro* durch die Gewinnung von embryonalen Stammzellen (ES-Zellen) auf unbegrenzte Zeit fortbestehen kann. Die zweite Eigenschaft, die ES-Zellen auszeichnet, ist die Selbsterneuerung. Diese Fähigkeit, weitere Stammzellen zu bilden, erfordert die Koordinierung der Zellproliferation und der Wahl der zellulären Identität (Orford and Scadden, 2008). Lange Zeit wurden murine ES-Zellen auf mitotisch inaktivierten Fibroblasten in einem Kulturmedium in Anwesenheit von fötalem Rinderserum und dem leukemia inhibitory factor (LIF) expandiert. Unter diesen Bedingungen sind die ES-Zellen metastabil und zeigen eine Heterogenität sowohl in ihrer Morphologie als auch in der Expression von wichtigen Pluripotenzfaktoren wie Oct4 und Nanog (Nichols and Smith, 2009). In Medien, die Serum und LIF enthalten, hängt die Pluripotenz und Fähigkeit zur Selbsterneuerung von dem Transkriptionsfaktor Myc ab (Smith et al., 2010). So wurde gezeigt, dass die Expression von Gata6, einem Masterregulator des primitiven Endoderms, durch Myc-Proteine gehemmt wird. Weiterhin kontrollieren sie den Zellzyklus, indem sie die Expression der mir-17-92-miRNAs regulieren. Arbeiten aus der Gruppe von Austin Smith konnten kürzlich zeigen, dass die Kultivierung in Abwesenheit von Serum und mit Supplementierung von zwei Inhibitoren (2i + LIF), die ES-Zellen im naiven Zustand der Pluripotenz halten können, dem sogenannten „ground state of pluripotency“. Dieser Zustand zeichnet sich durch eine größere Übereinstimmung zur inneren Zellmasse der Blastozyste aus (Ying et al., 2008). Die beiden zum Einsatz kommenden Inhibitoren hemmen dabei den Erk- sowie den Glykogen Synthase Kinase-3 (GSK3) Signalweg. Da mit verschiedenen Kulturbedingungen unterschiedliche Transkriptions- und epigenetische Eigenschaften in Maus ES-Zellen einhergehen (Marks et al., 2012), sollten im Rahmen dieser Arbeit die Expression und die Erfordernis der Myc-Proteine im Zusammenhang mit dem naiven Grundzustand der Pluripotenz untersucht werden.

Zur Untersuchung der c-Myc-Expression wurde eine transgene ES-Zelllinie erzeugt, die anstatt im endogenen c-Myc Lokus einen GFP-c-Myc-Reporter besitzt. Dabei stellte sich heraus, dass unter 2i-Bedingungen das c-Myc-Protein signifikant niedriger exprimiert war als unter Serumbedingungen. Nach conditioneller genetischer Deletion der c- und N-myc gene (Doppel-Knockout, dKO) arretiert der Zellzyklus der ES-Zellen. Charakteristischerweise geht eine solche Verlängerung der G1-Phase sowie verminderte Proliferation mit der Differenzierung von ES-Zellen einher. Im Gegensatz dazu zeigen Myc-dKO-ES-Zellen überraschend einen undifferenzierten Phänotyp. Dies zeigt sich morphologisch durch Bildung kleiner, undifferenzierter Kolonien sowie durch die fortwährende Expression der Pluripotenz-assoziierten Faktoren Oct4, Nanog und Sox2 sowohl auf RNA- als auch auf Proteinebene. Um die molekularen Eigenschaften von Myc-dKO-ES-Zellen zu untersuchen, wurden genomweite RNA-Sequenzierungen 24 und 96 Stunden nach dMyc-Deletion durchgeführt. Die Ergebnisse legen nahe, dass Myc nicht ausschließlich für die Regulation der Zellzyklusmaschinerie verantwortlich ist, sondern zusätzlich auf direkte und indirekte Weise für multiple Aspekte des Zellmetabolismus eine Rolle spielt. In Abwesenheit von c-Myc und N-Myc sind Gene der ribosomalen Biogenese sowie der Protein- und DNA-Synthese stark herabreguliert, was zu einem „metabolischen Ruhezustand“ der ES-Zelle führt.

Dieser metabolische Ruhezustand der ES Zellen *in vitro*, erinnerte an den Status der embryonalen Diapause. Diapause ist ein wenig verstandenes Phänomen eines reversiblen Arrests der embryonalen Entwicklung vor der Einnistung in die Uteruswand. In Mäusen kann es durch die Diapause zu einer verzögerten Implantation von Embryonen kommen, wenn das Muttertier noch einen vorangegangenen Wurf versorgt. Der Vergleich mit publizierten Daten zur globalen Genexpression von Embryonen in der Diapause (Hamatani et al., 2004) zeigt, dass c/N-Myc-deletierte ES-Zellen eine starke Ähnlichkeit mit der ruhenden Blastozyste haben. Dies ist charakterisiert durch die reduzierte DNA-Synthese und Zellteilung, niedriger metabolischer Aktivität und Aktivierung des Insulin-Signalweges (Given, 1988; Hamatani et al., 2004).

Diese Daten zeigen, dass c-Myc und N-Myc in Abwesenheit von Differenzierungssignalen für den gesamten Metabolismus und Synthesewege von ES

Zellen verantwortlich ist, während die Kontrolle der Pluripotenz durch andere Faktoren reguliert wird. Das Abschalten der Myc Aktivität ES Zellen in einen möglicherweise reversiblen metabolischen Ruhezustand, der dem Status von Diapause Embryos stark ähnelt.

3. Introduction

3.1 Stem Cells

Stem cells are defined by two main properties: self-renewal and capacity to differentiate into more mature cells (Reya et al., 2001).

Self-renewal is the process used by stem cells to generate more stem cells (He et al., 2009) with a mechanism that requires the coordination of cell proliferation and cell-fate choices (Orford and Scadden, 2008). A self-renewing stem cell can divide either symmetrically or asymmetrically to generate one or two daughter stem cells with a developmental potential similar to the mother cell. This self-renewal ability is necessary both during the embryonic development, to expand the number of stem cells, and throughout the adult life, to guarantee the perpetuation of the stem cell pool within adult tissues (He et al., 2009). Stem cells are able to differentiate into one or more specialized cell types: according to their differentiation potential, we can distinguish unipotent, multipotent or pluripotent stem cells.

According to their origin, stem cells can be categorized into embryonic and adult stem cells.

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the blastocyst, an embryonic structure that contains pluripotent cells with the unique capacity to differentiate into all the tissues of the adult organism. Although ES cells are not identical to ICM cells, they can be cultured *in vitro*, maintaining their ability to differentiate into all lineages and, when injected into blastocysts, they re-enter embryogenesis and participate in embryonic development (Nishikawa et al., 2007).

Adult stem cells play the essential role of sustaining the expansion of most tissues during postnatal life and to replace the cells lost upon injury (Dor and Melton, 2004). These stem cells are located in specific tissues such as the hematopoietic system, the intestine and the epidermis, and lie in a unique microenvironment, the stem cell niche, that controls their behavior and provides the factors necessary to regulate the balance between stem cell self-renewal and differentiation (Hsu and Fuchs, 2012).

Studying the biology of both embryonic and adult stem cells is essential to understand the mechanisms that regulate self-renewal and differentiation. This knowledge holds a great potential to be applied in cellular therapeutics for degenerative diseases (Orford and Scadden, 2008).

3.2 Embryonic stem cells

3.2.1 The origin of embryonic stem cells

The development of the mammalian embryo begins with the fertilization of the oocyte that is packed within the zona pellucida, a glycoprotein protective layer. After the formation of the zygote, a sequential round of divisions generates a cluster of cells known as blastomeres. Some of these cells remain apolar and occupy the inner part of the embryo, the so-called inner cell mass (ICM). At the same time, polar cells surround the ICM and form an outer, epithelial layer termed trophoblast (Johnson and Ziomek, 1981a, b). Trophoblast cells transfer fluid into the blastocyst to form a cavity, the blastocoel, and after cavitation the cells of the ICM develop into epiblast and hypoblast. The hypoblast is also known as “primitive endoderm”, and is an epithelium overlying the blastocoelic surface of the ICM (Nichols and Smith, 2012) (Figure 1). The newly formed epiblast is a cluster of 10–20 unspecialized cells. A single mouse epiblast cell, isolated at this stage and microinjected into another blastocyst, can contribute to all lineages (Gardner, 1998). Since each cell of the early epiblast is potentially able to generate the entire fetus, the pre-implantation epiblast can be considered as the developmental ground state (Nichols and Smith, 2009).

In 1981 Martin Evans was able for the first time to culture pluripotent stem cells from a mouse blastocyst (Evans and Kaufman, 1981). The establishment of the culture conditions able to support the growth of these embryonic stem (ES) cells was the result of more than 20 years of research on pluripotent cells present in malignant tumors called teratocarcinomas. In 1954, Stevens and Little established inbred strains of mice with high incidence of spontaneous testicular teratocarcinomas. These transplantable tumors originated from primordial germ cells of the fetal testes and, most interestingly, were composed of several types of

embryonic and adult tissues foreign to testes (Stevens and Little, 1954). Barry Pierce, in 1964, showed that the transplantation of single cells derived from teratocarcinomas could generate tumors containing a wide range of differentiated tissues (Kleinsmith and Pierce, 1964). These individual self-renewing pluripotent tumor cells were than defined embryonal carcinoma (EC) cells. In 1970, two independent groups were able to grow EC cells in culture (Kahan and Ephrussi, 1970; Rosenthal et al., 1970). These clonally isolated and *in vitro* expanded EC cells retained the capacity to form teratocarcinomas when injected into mice, although extensive passaging dramatically reduced their differentiation ability.

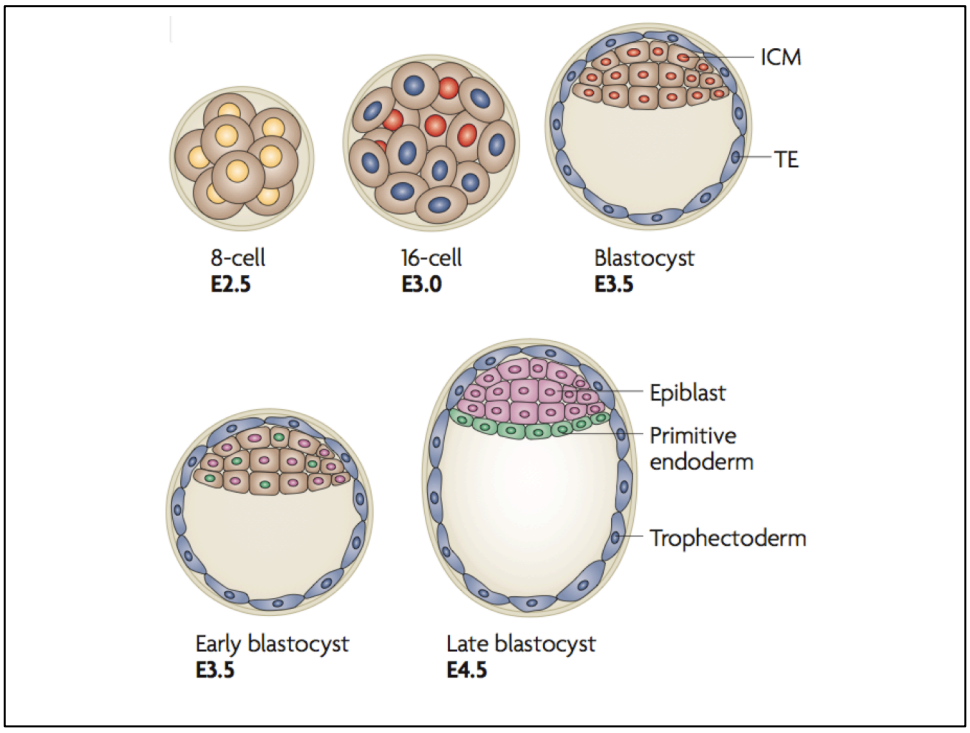

Figure 1: Lineage segregation in the blastocyst. The late blastocyst stage embryo (E4.5) contains three distinct lineage-restricted subpopulations: the trophectoderm, which mediates implantation and then expands to form the placenta; the primitive endoderm, that gives rise to the yolk sac; the early epiblast, which retains pluripotency and gives rise to all the somatic tissues and the germ cell lineage of the embryo. Modified from (Arnold and Robertson, 2009).

One of the proofs of the enormous similarity between EC cells and early embryonic cells was the capacity of some EC lines to participate in embryogenesis (Brinster, 1974; Papaioannou et al., 1975): when introduced into developing embryos, *in vitro* cultured EC cells could contribute to a variety of tissues, giving rise to chimeric mice.

Since EC cells were mostly aneuploid, probably as a consequence of selection during tumor growth and *in vitro* passage, they were not capable of proceeding through meiosis and produce mature gametes (Smith, 2001). Therefore several chimeric mice developed tumors and none of them was capable of germ-line transmission.

In 1970 there were the first, striking observations that early mouse embryos grafted into mice giving rise to teratocarcinomas (Solter et al., 1970; Stevens, 1970). The homology of EC cells with the early embryo became slowly clearer and the lessons learned from EC cells contributed to establish the conditions to culture the pluripotent stem cells from the embryos. In 1981 Martin Evans, Matthew Kaufman and Gale Martin were able for the first time to culture pluripotent cells directly from the mouse embryos on a feeder layer of mitotically inactivated fibroblasts (Evans and Kaufman, 1981; Martin, 1981), known to support the efficient establishment of EC cultures (Martin and Evans, 1975). These *in vitro* cultured pluripotent cells were named mouse embryonic stem (ES) cells and retained a completely normal, diploid karyotype. ES cells closely resembled EC cells in morphology, growth behavior, and markers expression (Smith, 2001) and, most extraordinary, once injected into early mouse embryos they could give rise to chimeras. Since a balanced set of chromosomes is necessary for meiosis, karyotypically normal ES cells could contribute to all the tissues of the chimaeras, including the germ-line (Bradley et al., 1984; Robertson et al., 1986). Since the establishment of the culture conditions able to support their growth, ES cell lines have been derived from pre-implantation epiblasts (Evans and Kaufman, 1981) but also from morulae and blastomeres at the eight-cell-stage (Tesar, 2005). However, until now there is no evidence showing that ES cells isolated from different developmental stages are molecularly or phenotypically different (Nichols and Smith, 2012). This suggests that embryonic cells are not frozen at the stage when they are put in culture but they may continue to follow a developmental program, and progress to the epiblast stage even if isolated from earlier stage embryos (Nichols and Smith, 2009).

In contrast to the limited number of pluripotent cells present in the ICM, *in vitro* cultured ES cells could be extensively expanded and manipulated. Making use of homologous recombination, Oliver Smithies and Mario Capecchi pioneered gene

targeting in ES cells (Capecchi, 2005; Smithies, 2005): they showed that an endogenous DNA sequence can be exchanged with a very similar exogenous DNA sequence to introduce specific genomic modifications in the recipient cell. *In vitro* cultured and genetically modified ES cells opened the era of *in vivo* mouse genetics.

3.2.2 ES cells pluripotency

The property of pluripotency is conferred to ES cells by the expression of three transcription factors Oct4, Nanog and Sox2, regarded as the intrinsic determinants of pluripotency. These proteins are expressed both *in vitro* in ES cells and *in vivo*, in the ICM of the blastocyst (Jaenisch and Young, 2008). Besides this core regulatory network, the gene expression program of *in vitro* cultured ES cells has been shown to more broadly depend on signal transduction pathways, chromatin regulators, and regulatory RNAs.

Intrinsic determinants of ES cells pluripotency

A core regulatory circuit consisting of the three transcription factors Oct4, Nanog and Sox2, is necessary to specify the undifferentiated state of ES cells. Functional studies have proven that these transcription factors bind to their own promoters, as well as the genes encoding the two other factors, establishing an autoregulatory circuitry (Boyer et al., 2005; Jaenisch and Young, 2008) (Figure 2) which might facilitate the maintenance of the pluripotent state (Alon, 2007).

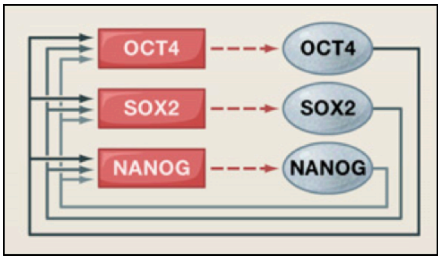

Figure 2: Auto-regulatory circuitry of the core ES cells pluripotency network. Oct4, Nanog and Sox2 form an interconnected auto-regulatory loop, binding their own promoters and the promoters of the other two master regulators of pluripotency. Modified from (Jaenisch and Young, 2008).

Oct4 is encoded by the *Pou5f1* gene and belongs to the Pit-Oct-Unc (POU) family of homeodomain proteins. Its expression is restricted to germ line cells, early embryos, ES cells and EC cells (Okamoto et al., 1990; Scholer et al., 1989a; Scholer et al., 1989b). *In vivo*, Oct4 is necessary in the establishment and maintenance of pluripotency: embryos lacking Oct4 develop to the blastocyst stage, but the cells of the ICM loose their pluripotency and differentiate into trophectoderm. For this reason, ES cell lines lacking Oct4 cannot be established (Nichols et al., 1998). Interestingly, altered levels of Oct4 expression can lead to divergent developmental programs in ES cells: while Oct4 repression induces loss of pluripotency and differentiation to trophectoderm, less than two fold increase in Oct4 expression causes differentiation into primitive endoderm and mesoderm (Niwa et al., 2000).

One of the key distinguishing roles of Sox2 appears to be the maintenance of Oct4 expression. As for Oct4, Sox2-null blastocysts fail to form a pluripotent ICM and mouse ES cells deficient for Sox2 differentiate into trophectoderm (Avilion et al., 2003). Enforced expression of Oct4 can rescue the differentiation phenotype of Sox deficient ES cells (Masui et al., 2007). The similarities between Oct4 and Sox2 knockout can be also attributed to the synergistic action of these two transcription factors in the regulation of several ES cells specific genes, as *Fgf4* (Yuan et al., 1995) and *Utf1* (Nishimoto et al., 1999).

After more than 10 years of studies in which Oct4 was regarded as the “master gene” of ES cells pluripotency, Nanog has been recently recognized as the third member of the core pluripotency network, as important as Oct4 in regulating the pluripotent state. Nanog was identified in 2003, independently by the groups of Austin Smith and Shinya Yamanaka, through a functional screening for genes conferring LIF-independent self-renewal (Chambers et al., 2003) and in an *in silico* differential expression analysis for genes exclusively expressed in mouse ES cells compared to differentiated tissues (Mitsui et al., 2003). Nanog-null embryos do not develop beyond implantation (Mitsui et al., 2003), since they lack a pluripotent ICM. Interestingly, *in vitro* deletion of both Nanog alleles in ES cells does not result in loss

of pluripotency (Chambers et al., 2007). Although Nanog deficient ES cells are more prone to differentiation, they can be maintained indefinitely in culture and contribute to somatic chimeras (Chambers et al., 2007). This observation suggests that Nanog might be necessary for the acquisition of pluripotency, but is dispensable once pluripotency has been achieved (Chambers et al., 2007; Silva et al., 2009).

An extended transcriptional network for pluripotency

Although Oct4, Sox2 and Nanog can be considered as master regulators of the core pluripotency network, the transcriptional network controlling ES cell pluripotency includes several more factors (Yeo and Ng, 2013).

Some of these regulators were identified by using genome-wide RNA interference (RNAi) screenings with loss of pluripotency as functional readout. These factors include Esrrb, Tbx3 and Tcl1 (Ivanova et al., 2006), the chromatin regulators Tip60-p400 (Fazzio et al., 2008) and SetDB1 (Bilodeau et al., 2009), and other important ES cells transcriptional co-factors as Cnot3 and Trim28 (Hu et al., 2009) and Paf1 (Ding et al., 2009).

More regulators of pluripotency were identified looking at protein-binding partners of the core pluripotency factors. Using affinity purification and mass spectrometry approaches, several Oct4 and Nanog associated proteins were discovered (Pardo et al., 2010; Wang et al., 2006), including Sall4 (Sakaki-Yumoto et al., 2006; Wu et al., 2006; Zhang et al., 2006) and Rif1 (Loh et al., 2006).

Another powerful tool to understand the basis of the pluripotency network is ChIP-seq analysis, consisting of chromatin immunoprecipitation coupled to massive DNA sequencing. This technology was applied to identify promoters of genes targeted by the core pluripotency factors (Nanog, Oct4 and Sox2), the somatic cell reprograming factors (Klf4 and c-Myc) and protein-interacting partners of Nanog and Oct4 (Dax1, Nac1, Zfp1 and Rex1) and provided a more comprehensive overview of the pluripotency network in ES cells (Kim et al., 2008).

Comparison of promoter occupation with global gene expression analysis has revealed that the core transcription factors occupy both the promoters of actively transcribed genes necessary to maintain the ES cell state, and the promoters of silent genes, whose expression would promote lineage commitment and cell differentiation (Boyer et al., 2005; Loh et al., 2006) (Figure 3).

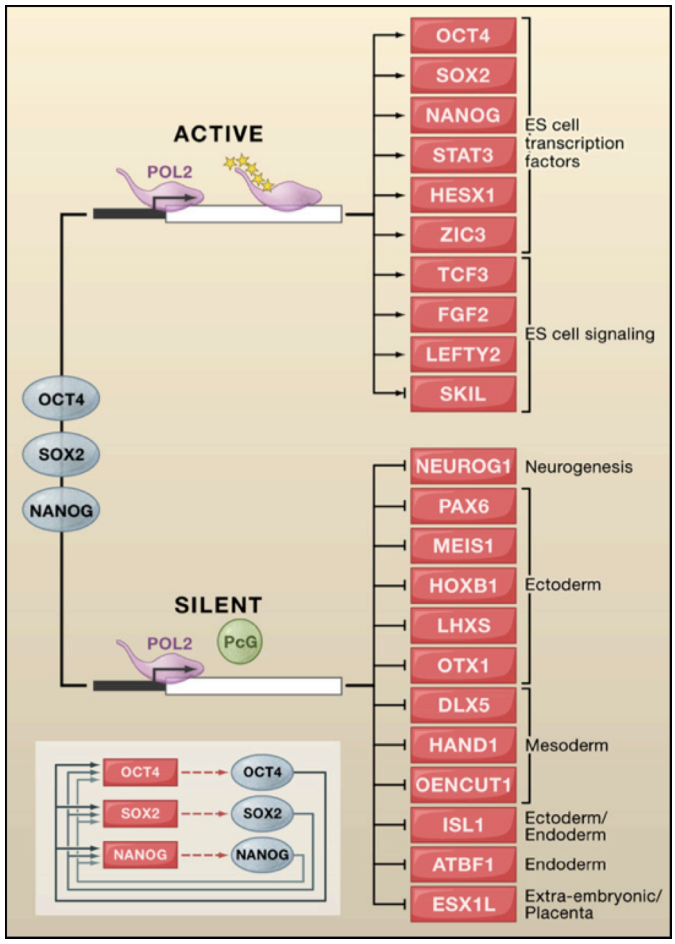

Figure 3: Model of the ES cell regulatory circuitry. Oct4, Sox2, and Nanog occupy actively transcribed genes, including transcription factors and signaling components necessary for pluripotency. At the same time, the three core pluripotency factors also occupy silent genes encoding transcription factors that, if expressed, would promote ES cells differentiation into different lineages. At this latter set of genes, RNA polymerase II (pol II) initiates transcription but does not produce complete transcripts since the polycomb (PcG) proteins prevent pol II from transitioning into a fully modified transcription elongation apparatus. Adapted from (Jaenisch and Young, 2008).

Although the transcription initiation apparatus is recruited to the promoters of developmental genes, in ES cells these genes are silent and poised for transcription.

This means that the RNA polymerase II (pol II) only generates short transcripts and pauses until pause-release factors and elongation factors allow further transcription (Young, 2011). In ES cells developmental genes are generally organized in bivalent domains, since they are characterized by nucleosomes with both histone H3K4me3, associated to gene activation, and H3K27me3, associated to gene repression (Jaenisch and Young, 2008). These bivalent domains have been proposed to silence developmental genes while keeping them poised for activation. Poised genes can be rapidly activated in response to differentiation stimuli (Bernstein et al., 2006).

Extrinsic determinants of pluripotency

When Martin Evans isolated and cultured ES cells for the first time, he made use of a culture medium containing fetal bovine serum (FBS) and a feeder layer of mitotically inactivated fibroblasts to support ES cells growth (Evans and Kaufman, 1981). Later, the medium was supplemented with the leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988) identified as the cytokine produced by the fibroblasts to sustain ES cells self-renewal (Niwa et al., 1998). Although self-renewal of *in vitro* cultured ES cells apparently depends on exogenous signals, it is important to keep into consideration that the requirement for a particular signal may be context-dependent (Boiani and Scholer, 2005) and signaling pathways affecting ES cells may not serve the same function in the developing embryo.

The LIF signaling cascade

LIF is a member of the IL6 family of cytokines. On the plasma membrane, the binding of LIF to its receptor (LIFR) causes the heterodimerization of the receptor with another transmembrane protein, the glycoprotein-130 (gp130) (Boulton et al., 1994). Upon LIF-induced receptor dimerization, the intracellular domain of the LIFR–gp130 heterodimer can recruit the Janus tyrosine kinase (JAK) (Boulton et al., 1994) and become phosphorylated, functioning as docking site for the signal transducer and activator of transcription-3 (STAT3). Receptor-bound STAT3 is phosphorylated on Tyr705 by JAKs before dimerizing and translocating to the nucleus where it acts to modulate transcription of target genes (Boiani and Scholer, 2005) (Figure 4). The

treatment of ES cells with LIF also induces the phosphorylation of the extracellular signal-regulated protein kinases, ERK1 and ERK2 (Burdon et al., 1999b), and increases the activity of the mitogen-activated protein kinase (MAPK) (Boeuf et al., 1997).

Although LIF is required *in vitro* to derive and preserve ES cells under conventional culture conditions (in the presence of serum), it is not clear if gp130-STAT3 signaling plays an analogous role *in vivo*. Indeed, mouse embryos lacking gp130 can develop to a stage subsequent to the establishment of pluripotent epiblast (Yoshida et al., 1996). This observation supports the hypothesis that alternative pathways might be involved in maintaining pluripotency *in vivo* and *in vitro*. Interestingly, if induced to enter diapause and to arrest at the late blastocyst stage, gp130 knockout embryos are unable to re-enter normal development (Nichols et al., 2001). Therefore, the responsiveness of ES cells to LIF may have its origins in the phenomenon of diapause.

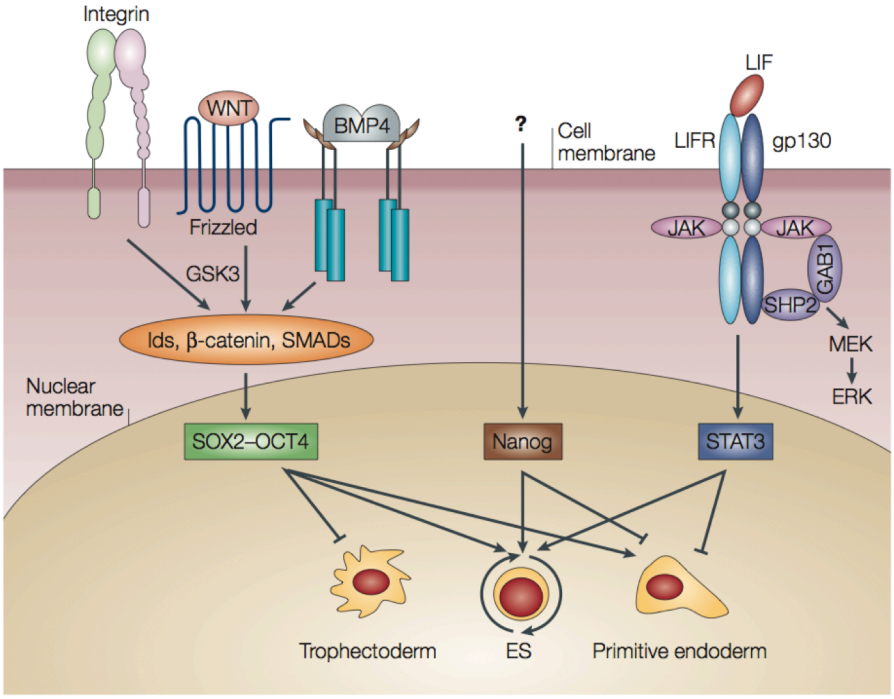

Figure 4: Signaling pathways controlling ES cells pluripotency. *In vitro* cultured ES cells can be exposed to several exogenous factors that are involved in nucleus-directed signaling pathways and modulate stem cell pluripotency. The leukemia inhibitory factor (LIF) exerts its effect by binding to the LIF receptor and activating STAT3. BMP4 contributes to the LIF cascade, enhancing the self-renewal and pluripotency of ESCs by activating the transcription factor SMAD4, which than activates members of the Id (inhibitor of differentiation) gene family (Ying et al., 2003). Adapted from (Boiani and Scholer, 2005).

3.2.3 Cell cycle of embryonic stem cells

Cell proliferation occurs through a series of events, collectively named as “cell cycle”. Classically, the cell cycle has been divided into four phases, characterized by DNA synthesis (S-phase) and mitotic segregation (M-phase), separated by gap phases to allow cell growth (G1 and G2) (Orford and Scadden, 2008). The transitions from G1 to S-phase and from G2 to M-phase are regulated by the presence of cell cycle “checkpoints” to assess, among other factors, the integrity of the genome.

In the absence of stimuli, cells can exit the cell cycle and enter a quiescent state, also termed G0. When cells are stimulated by mitogenic signals, they enter the cell cycle and go from an early G1-phase to a late G1-phase. This transition is also called “restriction point”, which represents a “point of no return” since cells in late G1-phase become independent on mitogenic signals to proceed through the cell cycle (Orford and Scadden, 2008) (Figure 5).

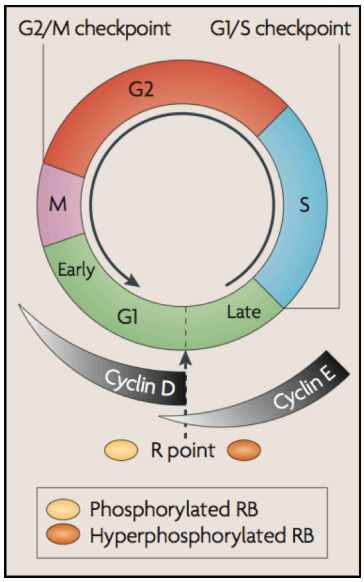

Figure 5: The eukaryotic cell cycle. The cell cycle consists of four distinct phases with the aim to replicate and transmit the genetic material to the daughter cells. DNA synthesis (S-phase) and cell division (M-phase) are separated by gap phases (G1 and G2) to allow cell growth. Adapted from (Orford and Scadden, 2008).

At the molecular level, cell cycle progression is highly regulated by the activity of cyclin-dependent kinases (CDKs) and cyclins. During the early G1-phase, mitogens activate cyclin D and CDK4 and 6, which drive the transition to the late G1-phase. In the late G1-phase, the cyclin E-CDK2 complex inactivates by hyperphosphorylation the Retinoblastoma (Rb) protein, which inhibits the E2F transcription factor (Hindley and Philpott, 2013). At this point, E2F can up-regulate targets necessary to enter into the S-phase (Hindley and Philpott, 2013).

In contrast to somatic cells, in *in vitro* cultured mouse ES cells the cyclin E-CDK2 complex is active throughout the cell cycle, and the Rb protein is continuously hyperphosphorylated (Burdon et al., 2002; Savatier et al., 1994). The absence of the restriction point and of a mitogen-dependent early G1-phase results in an extraordinary rapid cell cycle of around 11-16 hours (Orford and Scadden, 2008). Upon ES cells differentiation, the cell cycle is restructured, with lengthening of the G1-phase and decrease in rate of cell division (He et al., 2009).

3.2.4 Maintenance in culture of a naïve state of pluripotency

Cultured ES cells represent the immortalization of the early epiblast embryo (Nichols and Smith, 2009) and, retaining the ability to differentiate into all lineages, they represent an extraordinary tool to understand the molecular basis of developmental biology. To exploit the full potential of ES cells, it was essential to define the culture conditions able to stabilize them in a state that more closely resembles the naïve state of pluripotency in the mammalian embryo.

Since mouse ES cells were first described, they have been derived and maintained by using various combinations of feeder cells, conditioned media, cytokines, growth factors, hormones, fetal calf serum, and serum extracts (Ying et al., 2008). Nonetheless, ES cells cultured in serum-containing medium are exposed to signals promoting their differentiation and therefore exhibit great heterogeneity in morphology and in expression of core pluripotency factors such as Nanog (Chambers et al., 2007), Rex1 (Toyooka et al., 2008) and Stella (Hayashi et al., 2008; Wray et al., 2010). This heterogeneity suggested that mouse ES cells grown in serum may be in a

metastable and more developmentally advanced state that is not representative of the ground state of cells in the inner cell mass of the pre-implantation embryo (Guenther and Young, 2012).

Serum is not the only source of signals driving ES cells differentiation. Indeed, ES cells are capable to induce developmental pathways in an autocrine manner. Fgf4 is expressed both by epiblast and ES cells and it's a strong activator of the Erk pathway, which acts to induce ES cells differentiation (Kunath et al., 2007). The cytokine LIF, which promotes ES cells self-renewal by activating the transcription factor STAT3 (Niwa et al., 1998) also stimulates the MAPK/Erk pathway (Burdon et al., 1999a). Accordingly, genetic disruption or pharmacological inhibition of Erk signaling can reduce differentiation in ES cell cultures (Burdon et al., 1999a; Kunath et al., 2007). Partial inhibition of glycogen synthase kinase-3 (GSK3) has also been shown to enhance ES cells self-renewal (Sato et al., 2004; Ying et al., 2008) primarily through stabilization of intracellular β-catenin, which interacts with the T-cell factor 3 (Tcf3) and blocks its repression function on the pluripotency network (Wray et al., 2011).

On the basis of these observations, the group of Austin Smith has shown that extrinsic stimuli are dispensable for the derivation, propagation and pluripotency of mouse ES cells (Ying et al., 2008) and naïve pluripotency can be established *in vitro* in a serum-free culture by suppression of differentiation-inducing signals. The conventional ES cell culture medium composed by serum and LIF was initially substituted by a combination of three inhibitors, known as "3i", comprising an FGF receptor inhibitor (SU5402), an inhibitor of MAPK kinase (Mek) 1/2 (PD184352), and a GSK3 inhibitor (CHIR99021) (Ying et al., 2008). Since the FGF receptor is driving ES cells commitment primarily via the Erk pathway, the use of a Mek inhibitor (PD0325901) eliminates the need to block the FGF receptor. Hence, the 3i medium has finally evolved into 2i (Nichols and Smith, 2009; Wray et al., 2010). Mouse ES cells grown in the 2i + LIF more accurately represent the naive state of pluripotent cells in the inner cell mass of the blastocyst (Guenther and Young, 2012).

The exposure to different culture environments imposes distinctive transcriptional and epigenetic properties on mouse ES cells (Marks et al., 2012). Indeed, although ES cells grown in serum and 2i conditions have similar levels of transcripts for genes encoding key pluripotency factors (Pou5f1, Nanog, Sox2, Esrrb, Klf2, Klf4, and Tbx3), cells in 2i have decreased levels of several ectodermal and mesodermal specification genes.

3.3 The Myc family of transcription factors

In mammals, the Myc family of transcription factors comprises c-Myc, N-Myc and L-Myc (Adhikary and Eilers, 2005). c-Myc was the first one to be discovered, in 1982, as the human homologue of the oncogene *v-myc* of the avian myelocytomatosis virus (Vennstrom et al., 1982). N-Myc was identified shortly later, amplified in several human neuroblastoma cell lines and tumor samples (Kohl et al., 1983; Schwab et al., 1983). Finally, the third family member, L-Myc, was discovered for being deregulated in small cell lung cancer (Nau et al., 1985). Since their discovery, the Myc proteins have been implicated in the genesis of a variety of human tumors (Meyer and Penn, 2008) and enhanced Myc expression has been shown to contribute to several aspects of cancer biology (Adhikary and Eilers, 2005), including unrestricted proliferation (Eilers et al., 1991), inhibition of differentiation (Freytag and Geddes, 1992), cell growth (Iritani and Eisenman, 1999; Johnston et al., 1999), vasculogenesis (Baudino et al., 2002), reduction of cell adhesion (Arnold and Watt, 2001), metastasis (Pelengaris et al., 2002) and genomic instability (Felsher and Bishop, 1999).

Myc expression is frequently deregulated in tumorigenesis (Meyer and Penn, 2008). This is not only due to genetic changes at the Myc locus but also to alteration of one of the several mechanisms that target Myc expression and activity at transcriptional, post-transcriptional and post-translational levels (Levens, 2003; Sears et al., 1999).

Functional redundancy of Myc family members

Several studies indicate that Myc family members could have redundant functional properties during the early stages of embryogenesis, in particular c- and N-myc. Indeed, knockout of individual Myc genes is only lethal at embryonic day 10 or 12, as

a consequence of respectively c-myc or N-myc loss (Charron et al., 1992; Davis et al., 1993; Stanton et al., 1992). A very elegant study to address the potential of N-Myc to replace c-Myc was performed by generating NCR (N-C-Replacement) mice, where the c-myc coding region was replaced by the N-myc coding region by gene targeting (Malynn et al., 2000), and N-Myc protein was therefore expressed under the regulatory elements of the endogenous *c-myc* locus. Although homozygous c-myc$^{NCR/NCR}$ mice were healthy and fertile, they presented small abnormalities, as decrease in total body weight (Malynn et al., 2000). Additionally, lymphocytes or embryonic fibroblasts derived from these mice had a lower response to mitogenic stimulation, demonstrating that the c-myc^{NCR} allele can only partially compensate the loss of c-myc expression (Malynn et al., 2000).

Gene regulation by Myc-Max complexes

The Myc family members share significant homology in their genomic, RNA and protein sequences. All *myc* genes consist of three exons, of which only exons 2 and 3 are translated and encode for proteins that function primarily as nuclear transcription factors (Hurlin, 2013). The N terminus of the Myc proteins contains three highly conserved elements, known as Myc boxes I–III, which are required for Myc stability and for the interaction with other proteins. The C terminus consists of the basic region/helix–loop–helix/leucine-zipper (BR/HLH/LZ) domain (Figure 6).

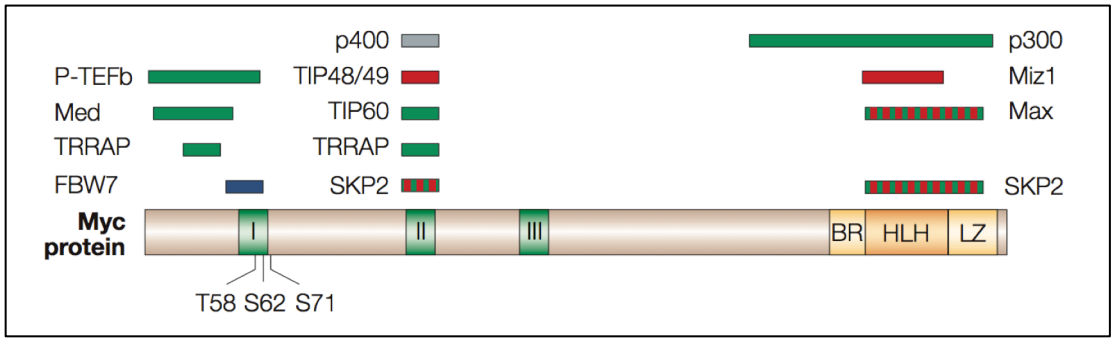

Figure 6: Domains of Myc and their binding proteins. The three Myc family members share at least 80% amino acid sequence homology in the functionally relevant domains Myc box I, Myc box II and basic region helix-loop-helix leucine-zipper (BR/HLH/LZ). These regions are essential to mediate the protein-protein interactions required for transcriptional regulation and for regulation of Myc stability. Modified from (Adhikary and Eilers, 2005).

The leucine zipper domain is a leucine-rich protein domain, present in a large class of dimeric transcription factors and mediates interactions with other proteins with a similar domain (Adhikary and Eilers, 2005).

Myc can both activate and repress transcription of its target genes through several mechanisms that include recruitment of transcription factors, chromatin modulating proteins, and DNA methyltransferases.

Myc does not bind the DNA on its own, but it interacts with a partner protein, Max, through the shared BR/HLH/LZ motif (Blackwood and Eisenman, 1991). Max is present in stoichiometric excess to Myc, and can also form homodimers or heterodimers with several related proteins, known as Mad1, Mad2, Mad3, Mad4 and Mnt (Adhikary and Eilers, 2005). All the dimers bind to the DNA through the same E-box sequence (CAC GTG), but whereas Myc-Max heterodimers activate the transcription of the target genes (Blackwood and Eisenman, 1991), Mad–Max and Mnt–Max heterodimers repress transcription (Ayer et al., 1993; Hurlin et al., 1997) (Figure 7).

Myc-Max heterodimers can activate the transcription through recruitment of histone and chromatin-modifying complexes that create a chromatin environment favorable for additional factors that cooperate in stimulating RNA polymerase activity (Figure 7). Indeed, Myc has shown to bind co-regulatory factors, such as TRRAP, which is a core subunit of the TIP60 and GCN5 histone acetyltransferase (HAT) complexes (McMahon et al., 1998) and other HAT complexes, as the CreB-binding protein (CBP) and p300 have also been described to interact with Myc (Vervoorts et al., 2003). Additionally, Myc recruits the INI1 subunit of the SWI/SNF chromatin-remodeling complex (Cheng et al., 1999).

Myc has also been found to recruit the cyclin-dependent kinase-9 (CDK9) subunit of the transcription elongation factor b (P-TEFb) (Kanazawa et al., 2003), which phosphorylates the carboxyl-terminal-domain (CTD) of the larger subunit of RNA pol II and is critical for the transition of pol II into a mature transcription elongation complex (Ni et al., 2008).

In addition to its ability to activate transcription, Myc can directly repress transcription of around 10-25% of its targets (Patel et al., 2004; Zeller et al., 2003). Myc-Max heterodimers can associate with the POZ domain/zinc finger transcription factor Miz1 (Seoane et al., 2001; Staller et al., 2001). Although Miz1 by itself acts as an activator of transcription, Myc-Miz1 complexes can repress transcription of Miz1 target genes, including cyclin-dependent kinase inhibitor 1A (*Cdkn1a*) encoding p21 (Wu et al., 2003) and *Cdkn2b*, encoding Ink4b (Seoane et al., 2001; Staller et al., 2001).

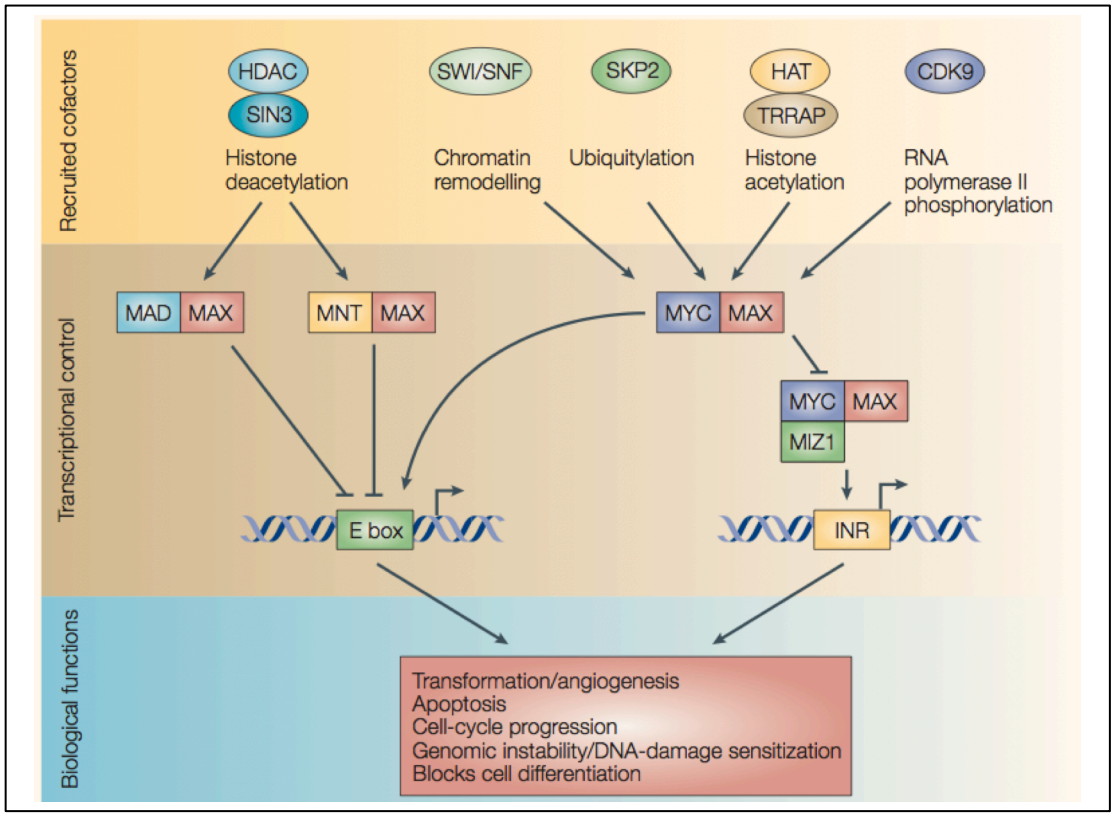

Figure 7: The Myc-Max network. Myc and its partner Max regulate several biological processes playing an essential role in tumorigenesis. Myc–Max heterodimers recruit cofactor complexes to E-box sequences near the transcriptional start site of target genes, including proteins involved in chromatin remodeling, ubiquitylation and histone acetylation. Myc cofactors include the cyclin-dependent kinase 9 (CDK9) that can directly phosphorylate RNA pol II and enhance transcriptional elongation. Max also dimerizes with Mad and Mnt family proteins, leading to recruitment of a scaffolding protein called SIN3, which in turn recruits histone deacetylases (HDACs) to mediate transcriptional repression of genes associated with E-box sequences. At certain promoters, Myc and Max interact with Miz1 to repress transcription. Adapted from (Patel et al., 2004).

Myc target genes

Since Myc was identified as a transcription factor with a crucial role in tumorigenesis, several different strategies have been developed to identify its target genes.

The first Myc target was described ten years after the identification of human Myc, when the Bishop's group developed a fusion of the human c-Myc to the hormone-binding domain of the estrogen receptor (ER) (Eilers et al., 1989). Myc–ER activation was shown to induce quiescent cells to enter and progress through the cell cycle (Eilers et al., 1989) and alpha-prothymosin (Ptma) (Eilers et al., 1991) and ornithine decarboxylase 1 (Odc1) (Wagner et al., 1993) were identified as transcriptional Myc targets. The role of Myc in promoting cell cycle progression was confirmed using other systems, as the Myc-ER^{TAM}, responsive to 4-hydroxytamoxifen (Littlewood et al., 1995), and Myc-null rat fibroblasts (Mateyak et al., 1997), leading to the identification of Gadd45a as a target of Myc repression (Marhin et al., 1997).

With the advent of the microarray technology, several groups performed large-scale analysis to identify Myc-regulated genes. Unexpectedly, all these studies produced long lists of putative Myc targets, showing often very little overlaps between each other. The more recent development of chromatin immunoprecipitation assay (ChIP), combined to high-throughput array technology (ChIP-chip), or with high-throughput nucleotide sequencing (ChIP-PET and ChIP-seq) has allowed the identification of the true Myc binding sites on the genome (Zeller et al., 2006). The integration of these data with expression array data allowed the development of a database of Myc-regulated genes (http://myccancergene.org) and the visualization of the complex Myc regulatory network (Dang et al., 2006). Recent estimates suggest that Myc can bind to approximately 10–15% of the genome from flies to humans and despite the functional range of specific genes altered, Myc consistently affects specific classes of genes that involve metabolism, protein biosynthesis, cell cycle regulation, cell adhesion and the cytoskeleton (Dang et al., 2006) (Figure 8).

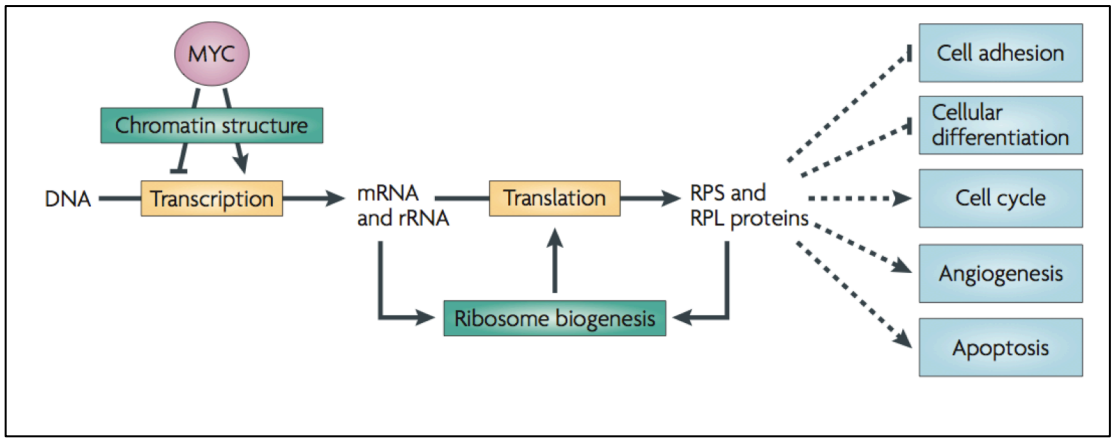

Figure 8: The pleiotropic function of the Myc family members. The transcription factor Myc participates in a variety of cellular processes by regulating the transcription of genes involved in cell adhesion, differentiation, cell cycle regulation, angiogenesis and apoptosis. Chromatin remodeling and regulation of ribosome biogenesis are thought to be two of the mechanisms used by Myc proteins to regulate globally gene expression. Adapted from (van Riggelen et al., 2010).

Cell cycle

The genes that consistently emerge and contain E-boxes bound by c-Myc in ChIP assays are cyclins D1 and D2, CDK4, and cyclin B1 (Bouchard et al., 2001; Fernandez et al., 2003; Hermeking et al., 2000; Menssen and Hermeking, 2002). c-Myc represses the CDK inhibitors p21 and p15INK4A through an interaction with the Miz-1 protein at the core promoter (Seoane et al., 2001; Wu et al., 2003) and genes involved in growth arrest such as Gadd45 (Barsyte-Lovejoy et al., 2004) and Gas1 (Lee et al., 1997).

Metabolism

Myc is a key regulator of many pathways of cell metabolism. Key enzymes of glucose metabolism, such as enolase A, hexokinase II, lactate dehydrogenase A, phosphofructokinase, and glucose transporter I, have been found to be Myc targets (Kim et al., 2004; Menssen and Hermeking, 2002; O'Connell et al., 2003; Osthus et al., 2000), supporting the hypothesis of a role of c-Myc in enhancing glucose uptake and glycolysis. Several genes involved in mitochondrial biogenesis and function are up-regulated in response to c-Myc overexpression in mammalian systems and in Drosophila (Morrish et al., 2003; Orian et al., 2003; Wonsey et al., 2002). Iron metabolism is also affected by Myc and several studies suggest that c-Myc functions

to increase the intracellular iron pool. Ferritin, Irp1, Irp2, and the transferrin receptor (Tfrc1) genes have been described as Myc targets (Morrish et al., 2003; Orian et al., 2003; Wonsey et al., 2002; Wu et al., 1999). Tfrc1, which is up-regulated by c-Myc, is a cell surface glycoprotein that transports iron-bound transferrin into the cell, where iron is then incorporated into enzymes that catalyze energy metabolism and DNA synthesis (Dang et al., 2006). Nramp1, which encodes a divalent cation transporter and removes iron from the cytosol, is repressed by c-Myc (Bowen et al., 2002). Finally, several genes involved in nucleotide synthesis and metabolism have also been shown to be important Myc targets (Dang et al., 2006).

Ribosome biogenesis and protein synthesis

Ribosome biogenesis is a complex and tightly coordinated process, which requires the synthesis, processing and assembly of several protein and RNA components (reviewed in (van Riggelen et al., 2010)).

Structural RNA components of the ribosome comprise the 5S, 5.8S, 18S and 28S rRNAs. The 18S, 5.8S and 28S rRNAs are encoded by ribosomal DNA (rDNA) in the nucleolus, where RNA pol I transcribes a single 47S rRNA precursor (pre-rRNA), which is then processed through endonucleolytic and exonucleolytic cleavages into 18S, 5.8S and 28S rRNA. The 5S rRNA is encoded in chromosome regions outside the nucleolus and is synthesized by RNA pol III. Ribosomal proteins are transcribed by RNA pol II, synthesized in the cytoplasm and then imported into the nucleus, where they are assembled into small and large ribosomal subunits (Figure 9) (van Riggelen et al., 2010). The small 40S ribosomal subunit contains one 18S rRNA and 32 ribosomal proteins, known as RPS proteins. The large 60S subunit is composed of one of each 5S, 5.8S and 28S rRNA and approximately 47 ribosomal proteins, known as RPL proteins. The 40S and 60S subunits are then exported into the cytoplasm, where they assemble with mRNA to form the 80S ribosome.

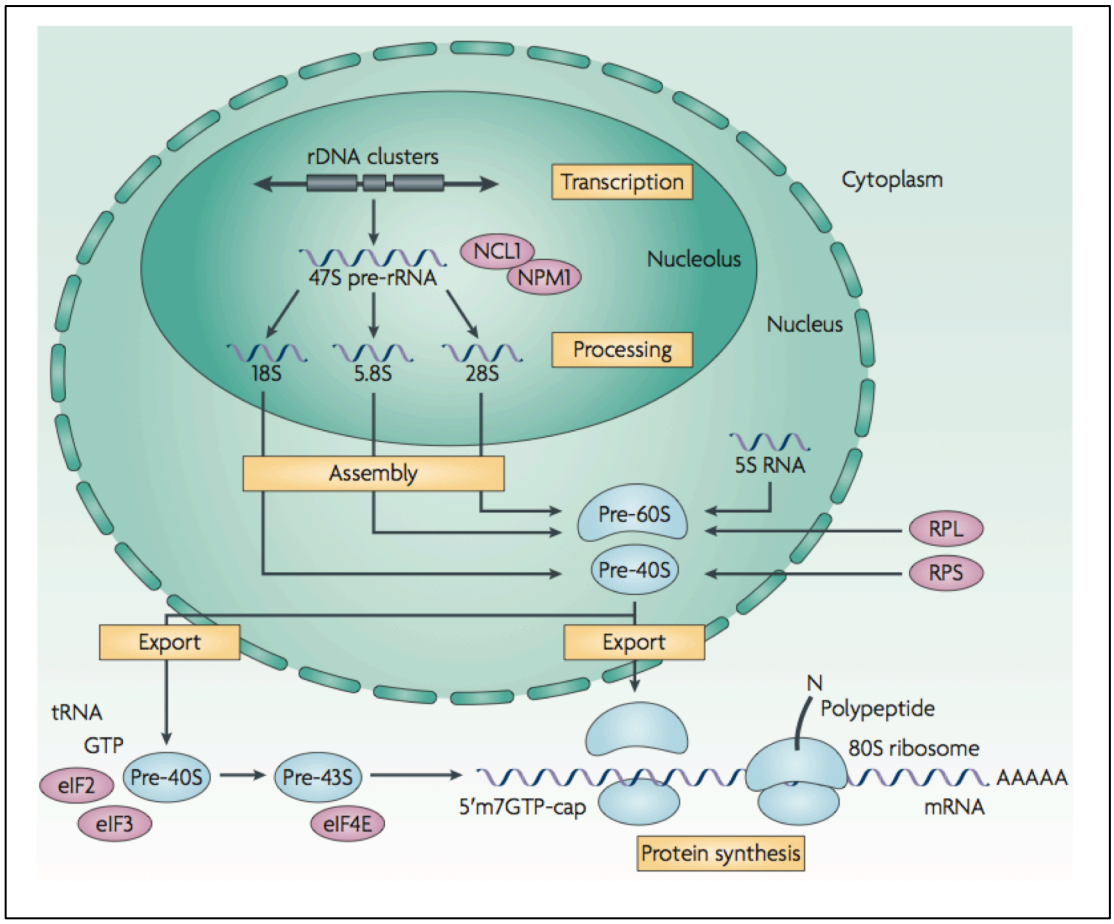

Figure 9: Overview of the ribosome biogenesis. The biogenesis of the ribosomes a highly coordinated process, consisting in the synthesis and import of ribosomal proteins into the nucleus, synthesis and processing of ribosomal RNA (rRNA), assembly of ribosomal proteins and transport of the mature subunits into the cytoplasm. Adapted from (van Riggelen et al., 2010).

Myc directly regulates the expression of several ribosomal protein, RNA components, and auxiliary factors that are required for ribosomal RNA (rRNA) processing, ribosome assembly, export of mature ribosomal subunits from the nucleus into the cytoplasm, as well as factors that control the initiation of mRNA translation (van Riggelen et al., 2010) (Figure 10).

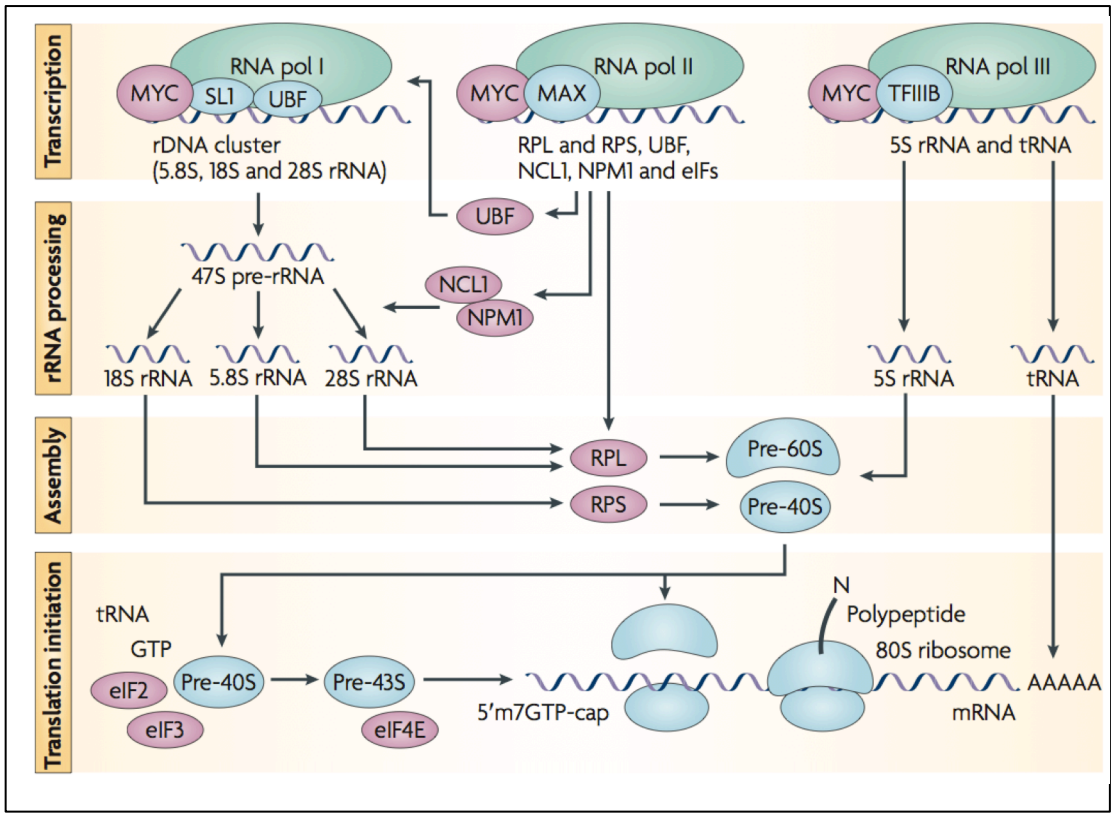

Figure 10: Myc controls multiple components of ribosome biogenesis. Myc regulates the RNA pol I-dependent transcription of 18S, 5.8S and 28S rRNAs. It facilitates rDNA transcription by enhancing the expression and/or recruitment of the RNA pol I cofactors upstream binding transcription factor (UBF) and selectivity factor 1 (SL1). The transcription of the 5S rRNA and tRNA, encoded on chromosome regions outside the nucleolus, is mediated by RNA pol III through the direct interaction of Myc with TFIIIB. Myc increases the transcription of many RPL and RPS proteins and translation initiation and elongation factors. Myc coordinates the transcription of genes that encode proteins required for the processing of rRNA precursors, contribute to ribosome assembly as well as the nuclear–cytoplasmic transport of mature ribosomal subunits. Such proteins include nucleolar protein 56 (NOP56), block of proliferation 1 (BOP1), fibrillarin (FBL), dyskerin (DKC1) nucleolin (NCL) and nucleophosmin (NPM1). NCL binds to the 47S pre-rRNA and is required for its cleavage into 18S, 5.8S and 28S rRNA in the nucleolus. NPM1 is responsible for the regulation rRNA processing, ribosomal protein stability and transport of ribosomal subunits into the cytoplasm. From (van Riggelen et al., 2010).

The role of Myc in stem cells

Making use of gene targeting in mice, the role of Myc proteins in development has been widely investigated: whereas L-myc knock-out mice develop normally (Hatton et al., 1996), embryos lacking c-myc die before E10.5 because of hematopoietic and placental defects (Dubois et al., 2008; Trumpp et al., 2001) and N-myc-deficient embryos die before E11.5 displaying neuroectodermal and heart defects (Charron et

al., 1992). Besides their role in development, several conditional genetic approaches have been used to reveal the function of the Myc family members in stem and progenitor cells (Laurenti et al., 2009) (Murphy et al., 2005).

ES cells, lacking only c-myc or N-myc show no obvious phenotype (Baudino et al., 2002; Sawai et al., 1991), probably as a consequence of the high functional redundancy between these two family members. Accordingly, deletion of both c- and N-myc in ES cells cultured in serum + LIF impairs maintenance of pluripotency and self-renewal (Smith et al., 2010). Indeed, under these culture conditions Myc proteins play a crucial role in repressing the expression of the primitive endoderm master regulator Gata6, and control the cell cycle by regulating the mir-17-92 miRNA cluster (Smith et al., 2010). In hematopoietic stem cells (HSCs), c-myc controls the balance between hematopoietic stem cells (HSCs) self-renewal and differentiation by regulating the interaction between HSCs and their niche (Wilson et al., 2004). Indeed, c-Myc-deficient HSCs divide without any measurable change in cell cycle, but fail to differentiate normally in a c-myc deficient environment (Wilson et al., 2004). Interestingly, simultaneous deletion of both c-myc and n-myc results in rapid loss of HSCs, due to impaired proliferation, differentiation and apoptosis (Laurenti et al., 2008). Finally, N-Myc loss results in decreased proliferation and increased differentiation of neural precursors (Knoepfler et al., 2002), demonstrating the important role that this gene plays in normal neurogenesis.

The role of Myc in reprogramming

Reprogramming refers to the process of altering the epigenetic program of a cell to change its developmental potential. Converting a somatic cell to a pluripotent cell has long been the goal of research, reflecting the enormous potential that such a technique could have in regenerative medicine.

In 1976, Miller and Ruddle demonstrated that thymocytes acquired pluripotency upon fusion with embryonal carcinoma (EC) cells (Miller and Ruddle, 1976). Indeed, if transplanted into nude mice, these cells were able to form teratomas consisting of tissues from all three germ layers. Reprogramming has also been achieved by transferring the nucleus of a somatic cell into an enucleated egg, a technique

referred to as somatic cell nuclear transfer (SSNT) (Wilmut et al., 1997) and by fusion of embryonic cells with somatic cells (Ambrosi and Rasmussen, 2005). However, both strategies have significant drawbacks: while nuclear transfer requires the use of oocytes and is highly inefficient, cell fusion results in the production of tetraploid cells which could not be used in cell replacement therapy because of their potential tumorigenic capacity (Ambrosi and Rasmussen, 2005).

In 2006, the Nobel laureate Shinya Yamanaka demonstrated that the overexpression of four transcription factors Oct4, Sox2, Klf4 and c-Myc could reprogram somatic cells to induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). iPS cells were first generated from mouse embryonic fibroblasts (MEFs) where the four transcription factors were introduced by retroviral transfection and ES-like cells were selected using the pluripotency-associated gene, Fbx15 (Takahashi and Yamanaka, 2006). Although these ES-like cells possessed pluripotency, as assessed by *in vitro* differentiation and teratoma formation, they were not able to generate germ-line competent chimeras and may therefore represent an incomplete state of reprogramming. Germ-line transmission was achieved later, by selecting iPS cells for the expression of endogenous Nanog (Okita et al., 2007) or Oct4 (Wernig et al., 2007), reflecting the requirement of the endogenous core regulatory network to establish true pluripotency (Silva and Smith, 2008).

Reprogramming is a gradual process that follows a defined series of molecular events (Brambrink et al., 2008; Stadtfeld et al., 2008) driving the repression of differentiation-specific genes and the activation of embryonic markers such as alkaline phosphatase (AP), SSEA1 and, in later stages, the pluripotency genes Nanog and Oct4. Several studies have been performed to understand how the four factors orchestrate the reprogramming process (Sridharan et al., 2009): Oct4 and Sox2 could act by promoting the expression of the endogenous Oct4, Sox2 and Nanog genes and activating the autologous pluripotency network. Once active, this network is expected to be self-promoting and to influence the activity of target genes whose expression or silencing is critical to the establishment of pluripotency. While the function of Oct4 and Sox2 in ES cells suggests how these factors could contribute to reprogramming, the role of c-Myc and Klf4 is less clear (Jaenisch and Young, 2008). Interestingly, reprogramming can be accomplished in the absence of c-Myc or Klf4,

albeit with a significant reduced efficiency and kinetics (Nakagawa et al., 2008; Wernig et al., 2008). The two oncogenes might confer somatic cells the immortal growth potential and rapid proliferative phenotype associated with ES cells (Yamanaka, 2008) and c-Myc overexpression might promote the switch to a more open chromatin state, allowing the reprogramming factors to more efficiently access genes necessary for reprogramming (Yamanaka, 2008). Interestingly, the group of Shinya Yamanaka has reported that L-Myc as well as c-Myc mutants (W136E and dN2) with lower transformation activity compared to wild type c-Myc, can promote more efficient and specific human iPS cells generation (Nakagawa et al., 2010). Therefore the role of Myc in reprogramming seems to be independent on its transformation property.

3.4 Epigenetic regulation of pluripotency

Epigenetic modifications are instructions that are imposed on the DNA sequence to provide an additional level of gene regulation (Hemberger et al., 2009).

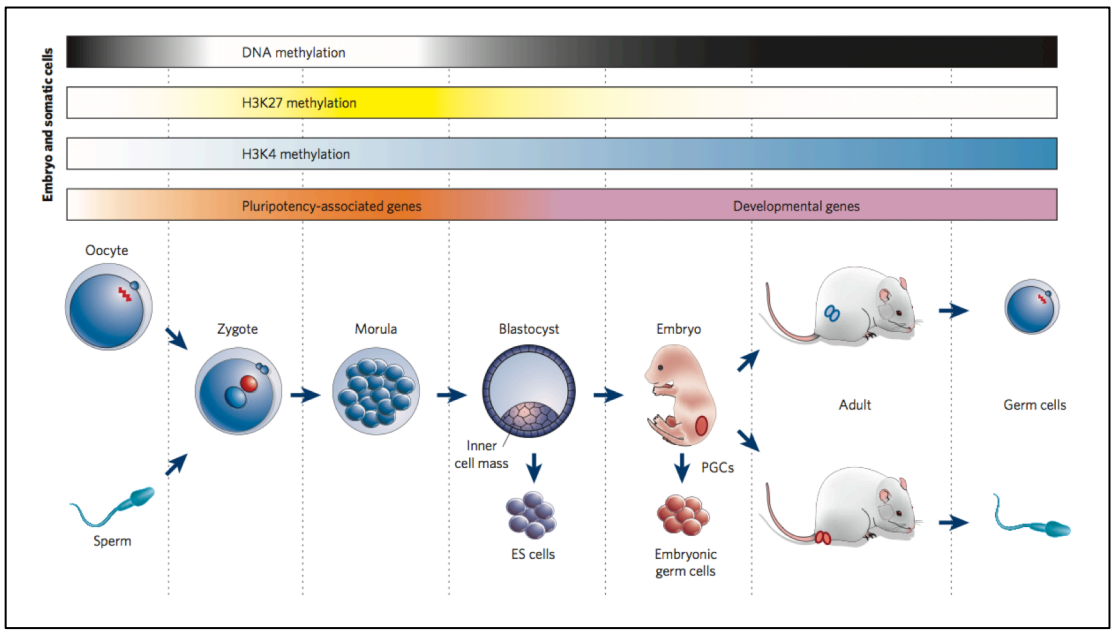

Figure 11: Epigenetic gene regulation during mammalian development. During the early phases of development, DNA methylation is erased, pluripotency-associated genes are expressed, while developmental genes are repressed by H3K27me3. During differentiation, pluripotency-associated genes are repressed as a result of DNA methylation while differentiation genes are expressed, and associated to H3K4me3. Modified from (Reik, 2007).

As development and differentiation proceed, the cells of distinct organs and tissues acquire specific epigenetic marks and possess unique gene expression profiles, being completely identical in their DNA sequences. In mammals, the most important epigenetic modifications include DNA methylation and covalent histones modifications (Hemberger et al., 2009) (Figure 11).

3.4.1 DNA methylation

DNA methylation is an epigenetic mark that refers to the addition of a methyl group to the fifth carbon of the base cytosine. In mammals, DNA methylation occurs predominantly in the context of CpG (C followed by G) dinucleotides and is catalyzed by enzymes named DNA methyltransferases (Dnmts).

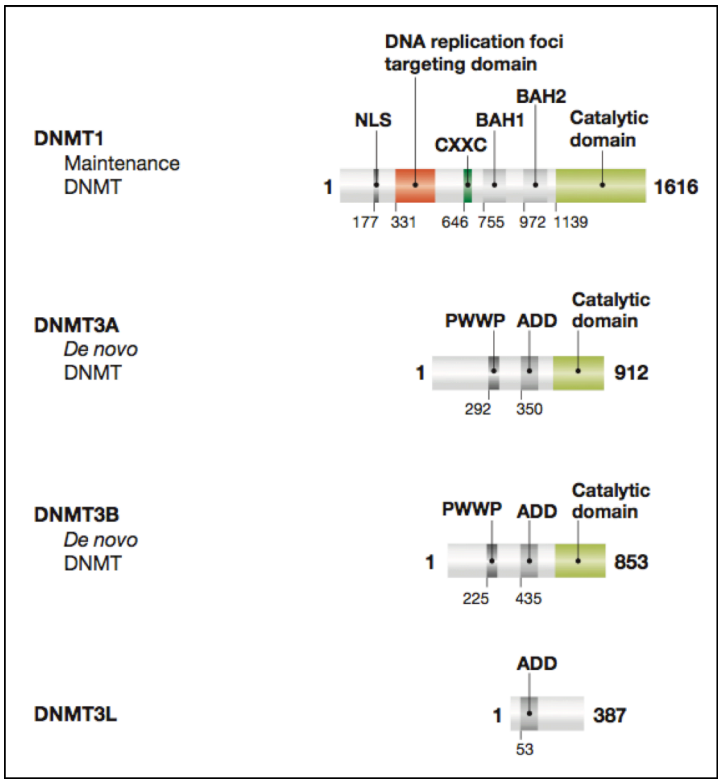

Figure 12: Structure of the human Dnmts. Schematic representation of the human Dnmt1, Dnmt3a, Dnmt3b and Dnmt3l structures and different domains. NLS, nuclear localization signal; CXXC, cysteine rich region; BAH, bromo-adjacent homology domain; PWWP, proline-tryptophan-tryptophan-proline domain; ADD, ATRX-DNMT3-DNMT3L-type zinc finger domain. Adapted from (Denis et al., 2011)

The family of Dnmts includes three catalytically active enzymes, Dnmt1, Dnmt3a and Dnmt3b and their close homologue, Dnmt3l, lacking catalytic activity (Meissner, 2010). Dnmt1 is primarily a maintenance methyltransferase that preserves methylation patterns during cell division. During S-phase it localizes to the DNA replication foci and preferentially methylates hemimethylated CpG dinucleotides (Denis et al., 2011). Dnmt3a and Dnmt3b are responsible for *de novo* DNA methylation.

All the Dnmts are essential for mammalian development. Loss of Dnmt1 results in embryonic lethality around E8.5-9, and Dnmt1 mutant embryos retain only one-third of the normal amount of DNA methylation (Li et al., 1992; Meissner, 2010). Dnmt3b mutant embryos do not develop to term and show multiple developmental defects already after E9.5 (Okano et al., 1999). Dnmt3a knockout mice die around 1 month after birth (Okano et al., 1999), while homozygous Dnmt3l mice are viable, although males are sterile and heterozygous offspring of homozygous females die due to imprinting defects (Bourc'his et al., 2001). Both Dnmt3a and Dnmt3l seem to have an essential role in maternal imprinting (Bourc'his et al., 2001; Kaneda et al., 2004). Albeit the complete absence of CpG methylation, ES cells deficient for all three methyltransferases Dnmt1, Dnmt3a and Dnmt3b are able to self-renew and maintain the molecular signature of pluripotency (Tsumura et al., 2006). Although DNA methylation is not required for maintenance or establishment of ES cells pluripotency, it has a crucial role in commitment: in the absence of DNA methylation, differentiation is almost completely inhibited since ES cells fail to upregulate germ-layer-associated markers and do not efficiently silence the pluripotency network (Jackson et al., 2004).

Recent studies have shown that different culture conditions are associated with profound changes in global DNA methylation: interestingly, naïve ES cells cultured in 2i conditions are characterized by lower expression of the *de novo* DNA methyltransferases Dnmt3a, Dnmt3b and Dnmt3l and are associated to global DNA hypomethylation (Leitch et al., 2013).

3.4.2 Histone modifications

DNA is packaged within the cell in a structure termed chromatin. The nucleosome is the fundamental unit of chromatin and is composed of an octamer of four core histones (H3, H4, H2A, H2B) around which are wrapped 147 base pairs of DNA (Kouzarides, 2007). The core histones that make up the nucleosome are subject to a broad range of posttranslational modifications that can have both repressive and activating functions and include acetylation, methylation, phosphorylation, ADP ribosylation and ubiquitylation. Histone modifications occur at specific positions primarily within the amino-terminal histone tails (Bernstein et al., 2007). Among the best-characterized mediators of histone modifications there are the protein complexes of the polycomb (PcG) and trithorax (trxG) groups (Schuettengruber et al., 2007), catalyzing tri-methylation of histone H3 respectively on lysine 27 (H3K27me3) and lysine 4 (H3K4me3) (Hemberger et al., 2009). H3K4me3 positively regulates transcription by recruiting nucleosome remodeling enzymes and histone acetylases (Pray-Grant et al., 2005; Santos-Rosa et al., 2003; Sim and Denlinger, 2013; Wysocka et al., 2005), while H3K27me3 is a negative regulator of transcription since it induces a more compact chromatin structure (Ringrose et al., 2004). In ES cells several genes encoding for developmental transcription factors are associated with both the repressive H3K27me3 and with the active H3K4me3 (Bernstein et al., 2006).

This particular chromatin modification pattern is known as “bivalent domain”, and is thought to be important in ES cells to silence developmental genes while keeping them poised for activation (Bernstein et al., 2006).

4. Aim of the thesis

The main goal of this thesis was to elucidate the role of the Myc family of transcription factors in naïve mouse embryonic stem (ES) cells cultured in 2i + LIF medium. To genetically address this question, we derived ES cell lines carrying a Cre/loxP system to conditionally delete both c-myc and N-myc. We then investigated the effect of Myc double knock-out (dKO) on self-renewal and pluripotency by analyzing the cell cycle profile and the expression of the core ES cells pluripotency factors upon Myc activity loss. Considering that Myc proteins can influence a variety of cellular processes by globally regulating gene expression, one of our aims was to determine the molecular landscape of Myc dKO ES cells. To answer this question, we performed whole transcriptome analysis by next-generation sequencing (RNA-seq). Finally, to compare c-Myc protein expression in naïve ES cells, cultured in 2i + LIF medium, and metastable ES cells, cultured in serum + LIF medium, we derived ES cells from mice expressing a c-Myc-GFP reporter protein introduced in-frame into the endogenous *c-myc* locus.

5. Results

5.1 The effect of Myc loss in naïve ES cells

In vitro cultured ES cells have been the focus of an enormous number of studies to elucidate the mechanisms of self-renewal and pluripotency. However, ES cells are derived from a cell type that exists only transiently in the early embryo and *in vitro* are maintained in an artificial environment. Recent studies have shown that the conventional medium used to culture ES cells, containing serum and the cytokine LIF, exposes these stem cells to a multitude of extracellular signals promoting their differentiation. ES cells cultured in "serum + LIF" are therefore metastable since exhibit great heterogeneity in morphology and in the expression of core pluripotency factors such as Oct4 (Nichols and Smith, 2009), Nanog (Chambers et al., 2007), Rex1 (Toyooka et al., 2008) and Stella (Hayashi et al., 2008; Wray et al., 2010). Recently, the group of Austin Smith has defined a new culture condition able to stabilize mouse ES cells in a naïve state of pluripotency, that more closely recapitulates the pluripotent state in the early mammalian blastocyst (Leitch et al., 2013; Marks et al., 2012; Yamaji et al., 2013; Ying et al., 2008). Naïve ES cells can be maintained in a serum-free medium, supplemented with LIF and in the presence of two small molecules, PD0325901 and CHIR99021, inhibiting respectively MEK and GSK3β. These novel culture conditions are therefore referred to as "2i + LIF". The role of several factors involved in ES cells biology has been studied in serum-containing cultures. Taking into account that ES cells are more homogenous in 2i and differ from those cells cultured in serum both on a gene expression and epigenetic level (Marks et al., 2012), it is necessary to re-evaluate the role of several key pluripotency factors in the absence of differentiation signals. The role of the Myc family members in ES cells grown in serum +LIF has been broadly described. Separate deletion of either c- or N-myc seems to have no effect on mouse ES cells: indeed, embryos lacking either c- or N-myc die at E10.5 or E11.5 respectively (Charron et al., 1992; Davis et al., 1993; Sawai et al., 1991; Trumpp et al., 2001). These results could be explained taking into consideration that c-myc and N-myc are highly redundant (Malynn et al., 2000) and one family member might compensate the absence of the

other in ES cells. Recent publications have shown that loss of both c- and N-myc in metastable ES cells impairs self-renewal and induces rapid differentiation towards primitive endoderm (Smith et al., 2010; Varlakhanova et al., 2010). Elucidating the function of c- and N-myc in the naïve state of pluripotency is the aim of this project.

5.1.1 Derivation of ES cell lines for conditional deletion of c- and N-myc

In collaboration with the "transgenic service" of the German Cancer Research Center (DKFZ), we derived ES cell lines from c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$ mice.

The REYFP^{flox} is an allele reporting Cre recombinase activity (Srinivas et al., 2001): a loxP-flanked stop codon blocks the expression of the EYFP protein and upon Cre-mediated recombination, the stop codon is excised, resulting in constitutive EYFP expression from the ubiquitously active *Rosa26* promoter (Srinivas et al., 2001) (Figure 34).

E2.5 days morulas were cultured for the first two passages on a feeder layer of γ-irradiated MEFs, in "serum + LIF" medium that was additionally supplemented with the two small molecule inhibitors PD0325901 and CHIR99021 to support a more efficient ES cell derivation. Under these conditions we generated 6 different monoclonal ES cell lines where both c- and N-myc can be conditionally deleted in the presence of Cre recombinase activity. These lines were further maintained in serum-free, "2i + LIF" medium. Lines B8 and D3 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$) were used to investigate the effect of both c- and N-Myc loss on ES cell self-renewal and pluripotency. The genetic identity of these ES cell lines was confirmed via PCR (Figure 15.B).

5.1.2 Teratoma Assay

ES cells are by definition pluripotent, since they are capable to differentiate into all cell lineages of the body. The teratoma assay is one of the standard techniques to test the differentiation potential of embryonic stem cells. Once injected into an immunodeficient mouse, ES cells are exposed to a complex mixture of factors able to support their proliferation and differentiation into teratomas, non-malignant tumors

comprised of tissues derived from all three germ layers: ectoderm, mesoderm and endoderm (Wesselschmidt, 2011).

In order to prove the multi-lineage differentiation capacity of the newly derived ES cell lines, we injected subcutaneously $2x10^6$ cells into immunodeficient NSG mice. 8 weeks after injection, when the tumors were visible and around 1.5 cm in diameter, the mice were euthanized and the tumors surgically removed and processed for histopathological analysis (Figure 13). Hematoxylin and Eosin (H&E) staining of teratomas showed that ES B8 cells were able to differentiate into skin-like structures with keratin deposition (ectoderm, Figure 13.A and D), cartilage (mesoderm, Figure 13.B and E) and gastrointestinal epithelium (endoderm, Figure 13.C).

The capability to differentiate into tissues derived from the three germ layers confirms the broad pluripotent potential of the newly derived ES lines.

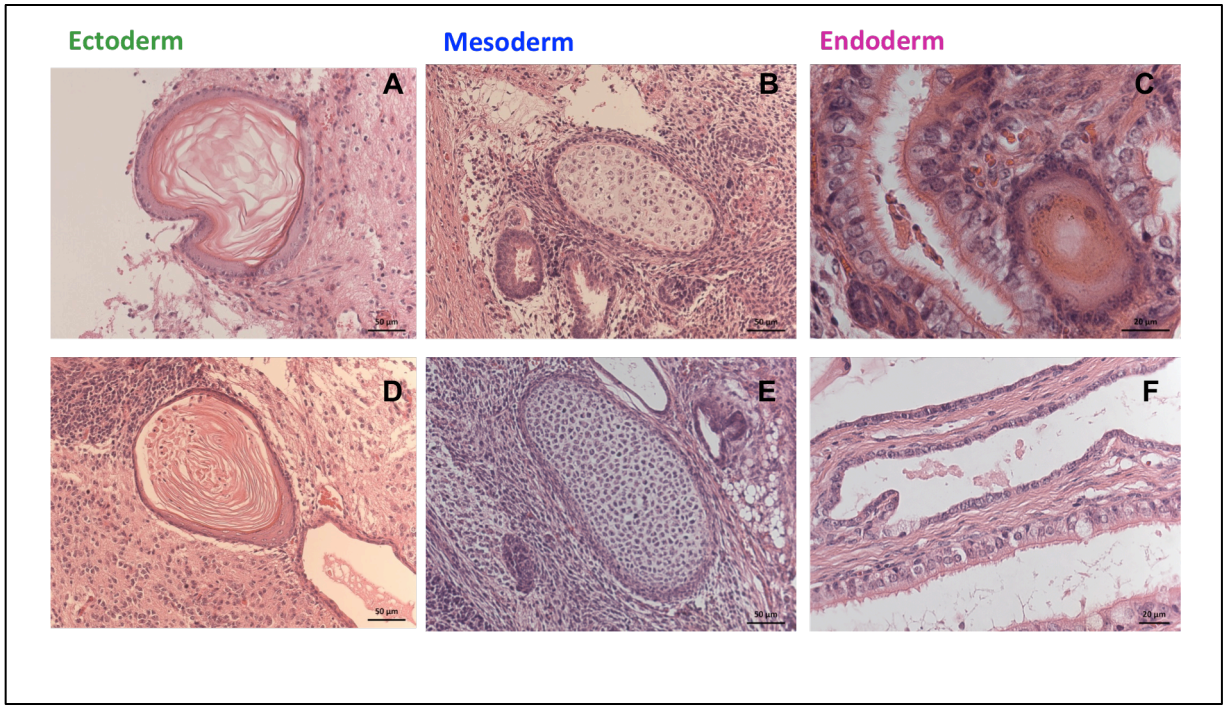

Figure 13: Histology of teratomas derived from c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$ mouse ES cells. H&E staining of representative structures of ectodermal (**A,D**), mesodermal (**B,E**) and endodermal (**C,F**) origin derived from teratomas developed from the ES B8 line. Scale bars (A,B,D,E) 50µm; (C,F) 20µm.

5.1.3 Deletion of c-myc and N-myc in mouse ES cells

The schematic representation of the strategy used to genetically delete c- and N-myc in ES cells is shown in Figure 14. In summary, the cells were transiently transfected with the pbS513 plasmid (Le et al., 1999) expressing the Cre recombinase under the control of the elongation factor 1-alpha (EF1α) promoter, highly active in mouse ES cells. The REYFP^{flox} reporter allele was used to identify those cells where Cre was expressed. 24 hours after transfection, EYFP+ cells were FACS sorted (Figure 15.A) and cultured in 2i + LIF medium. After 72h, more than 120 single colonies were mechanically picked, expanded as single clones and genotyped (Figure 15.B).

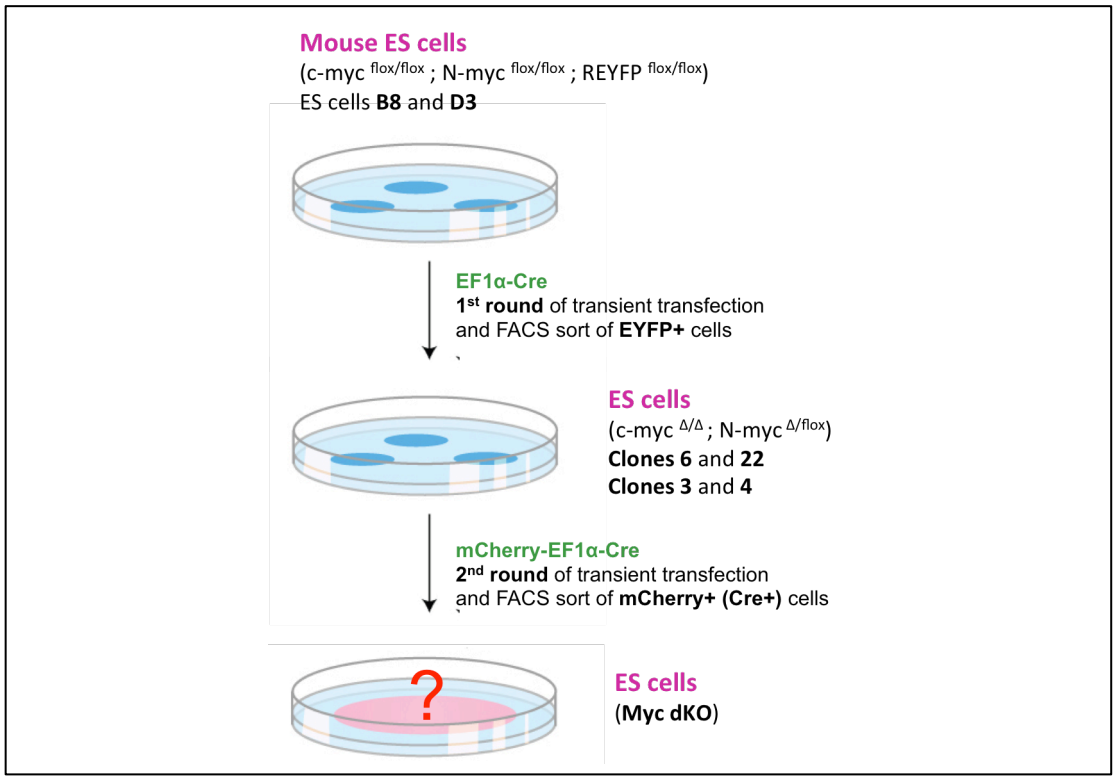

Figure 14: Experimental strategy to delete c-myc and N-myc in mouse ES cells. To obtain Myc dKO ES cells, B8 and D3 ES cells (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$) were transfected with an EF1α-Cre plasmid (pbS513) and EYFP+ cells were sorted 24 hours after transfection. After sort, single ES cell colonies were picked, separately grown and genotyped. Clones 3, 4, 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were than transiently transfected with a mCherry-Cre plasmid to express the Cre recombinase and delete the last N-myc allele.

All the clones that could be further expanded had only partially deleted myc, since they were always carrying at least one residual N-myc or c-myc allele. In Figure 15.B are shown the genotyping PCRs of clones 6 and 22 (derived from the B8 line) and

clones 3 and 4 (derived from the D3 line) carrying a residual N-myc allele (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$). Clone 11 (derived from the D3 line) had only c-myc deletion (c-myc$^{\Delta/\Delta}$; N-myc$^{flox/flox}$). In 2i + LIF medium, ES cell lines lacking both c-myc alleles were morphologically indistinguishable from the parental lines (data not shown). The observation that it was not possible to derive and expand ES cell clones in which the two alleles of both c- and N-myc have been fully deleted indicates a strong negative selection against these cells.

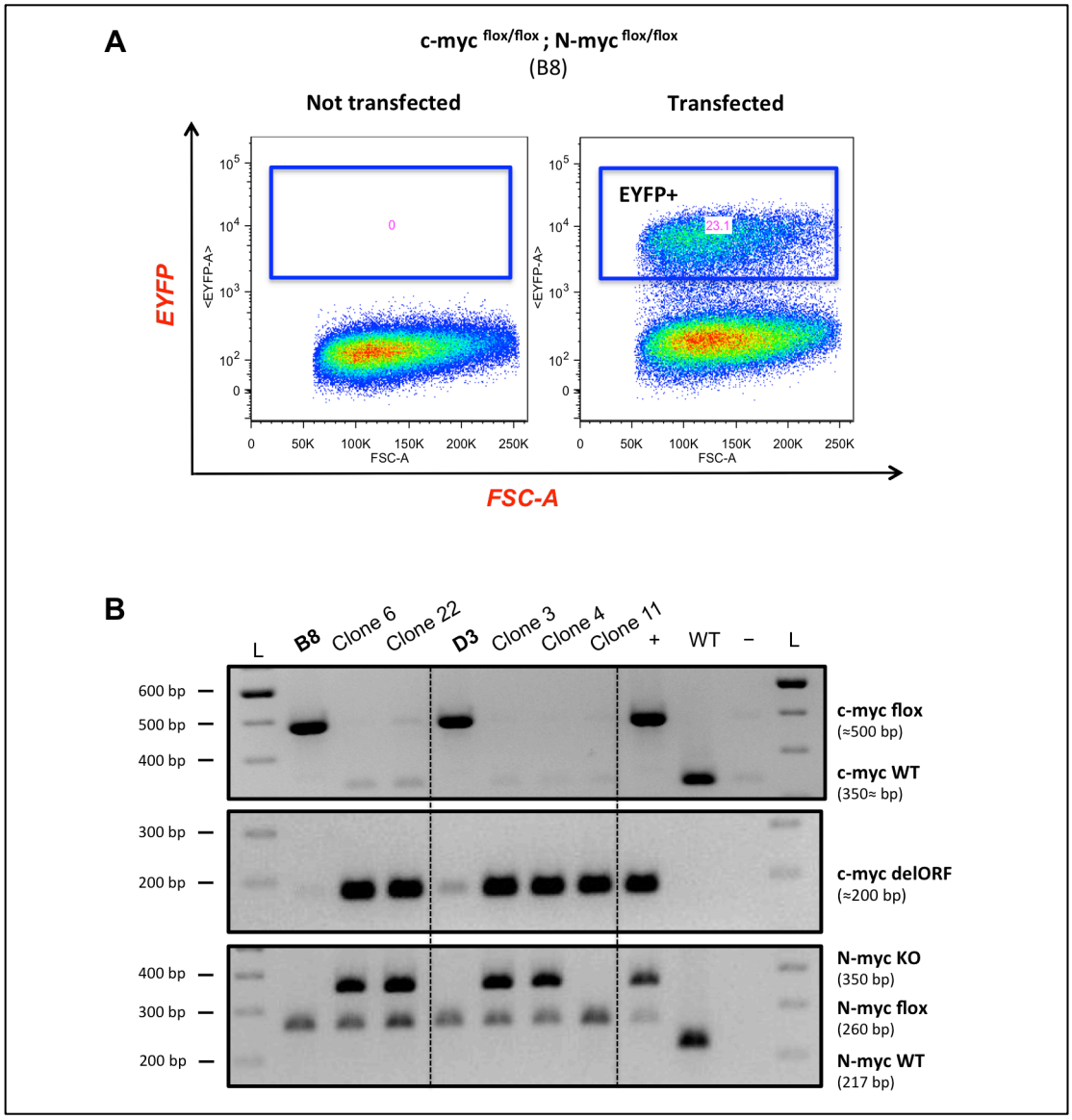

Figure 15: Deletion of c-myc and N-myc in mouse ES cells. B8 and D3 ES cells (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$) were transfected with an EF1α-Cre plasmid (pbS513) and EYFP+ cells were FACS sorted 24 hours after transfection. After sort, single ES cell colonies were picked, grown separately and genotyped. (**A**) Representative scheme for FACS sorting of EYFP+ ES cells 24 hours after Cre induction. (**B**) Genotyping PCR of B8 and D3 ES cells and the clones that were derived upon Cre-mediated myc deletion. L (100 bp DNA ladder); + (positive control); - (negative control, water); WT (wild type).

In order to obtain a population enriched for Myc dKO ES cells, we used those clones carrying only one residual N-myc allele for a second round of Cre induction. Since these cells constitutively express the EYFP reporter, to delete the last N-myc allele, clones 6 and 22 were transfected with the mCherry-Cre plasmid expressing a Cre-mCherry fusion protein under the control of the EF1α promoter (Figure 14).

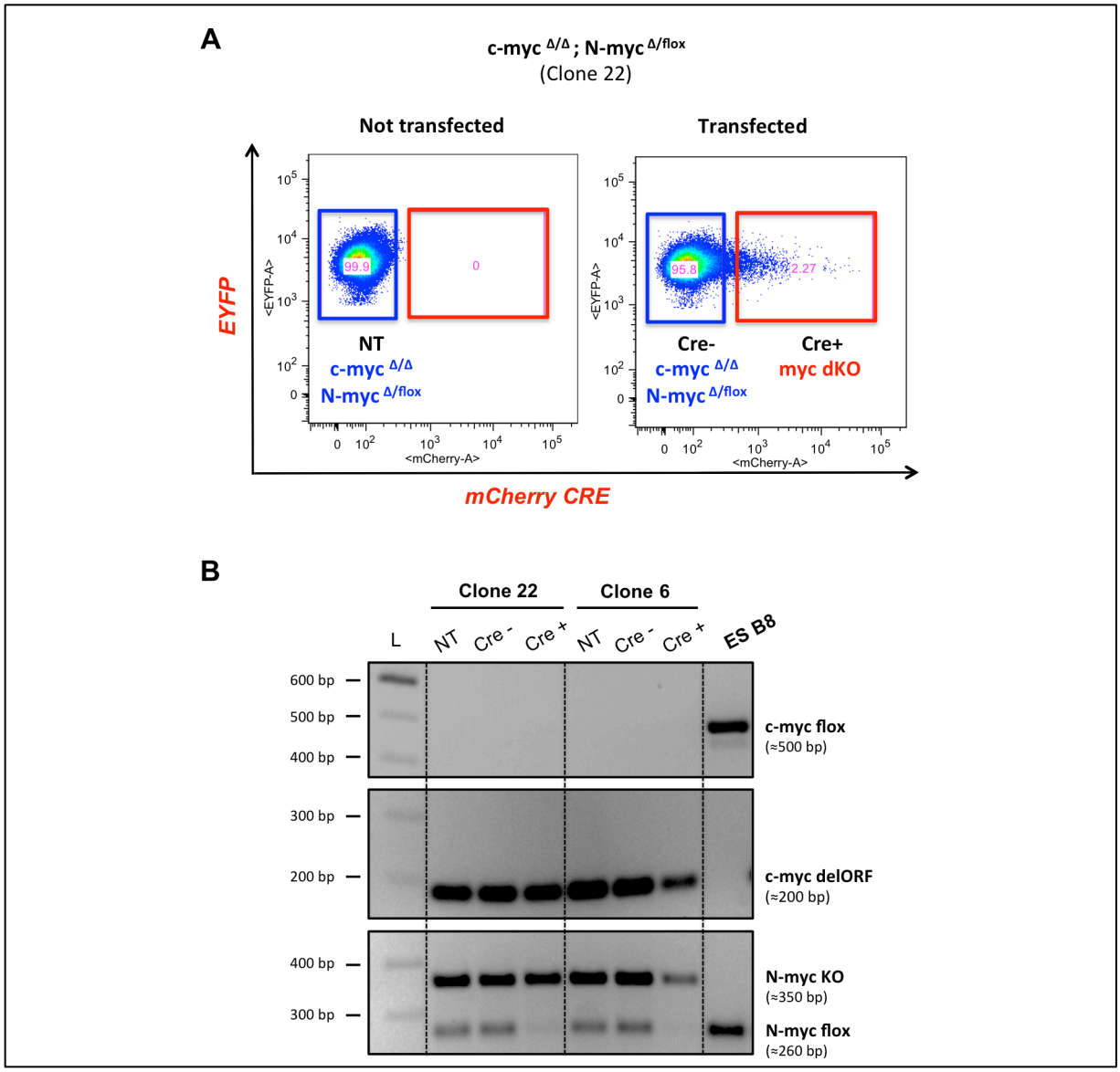

Figure 16: Deletion of the residual N-myc allele. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were transfected with a mCherry-Cre plasmid and mCherry+ (Cre+) and mCherry- (Cre-) cells were sorted 24 hours after transfection. (**A**) Representative scheme for FACS sorting of mCherry+ and mCherry- ES cells 24 hours after Cre induction. (**B**) Immediately after sort, ES cells were lysed and used for DNA isolation and genotyping. L (100 bp DNA ladder); NT (not transfected).

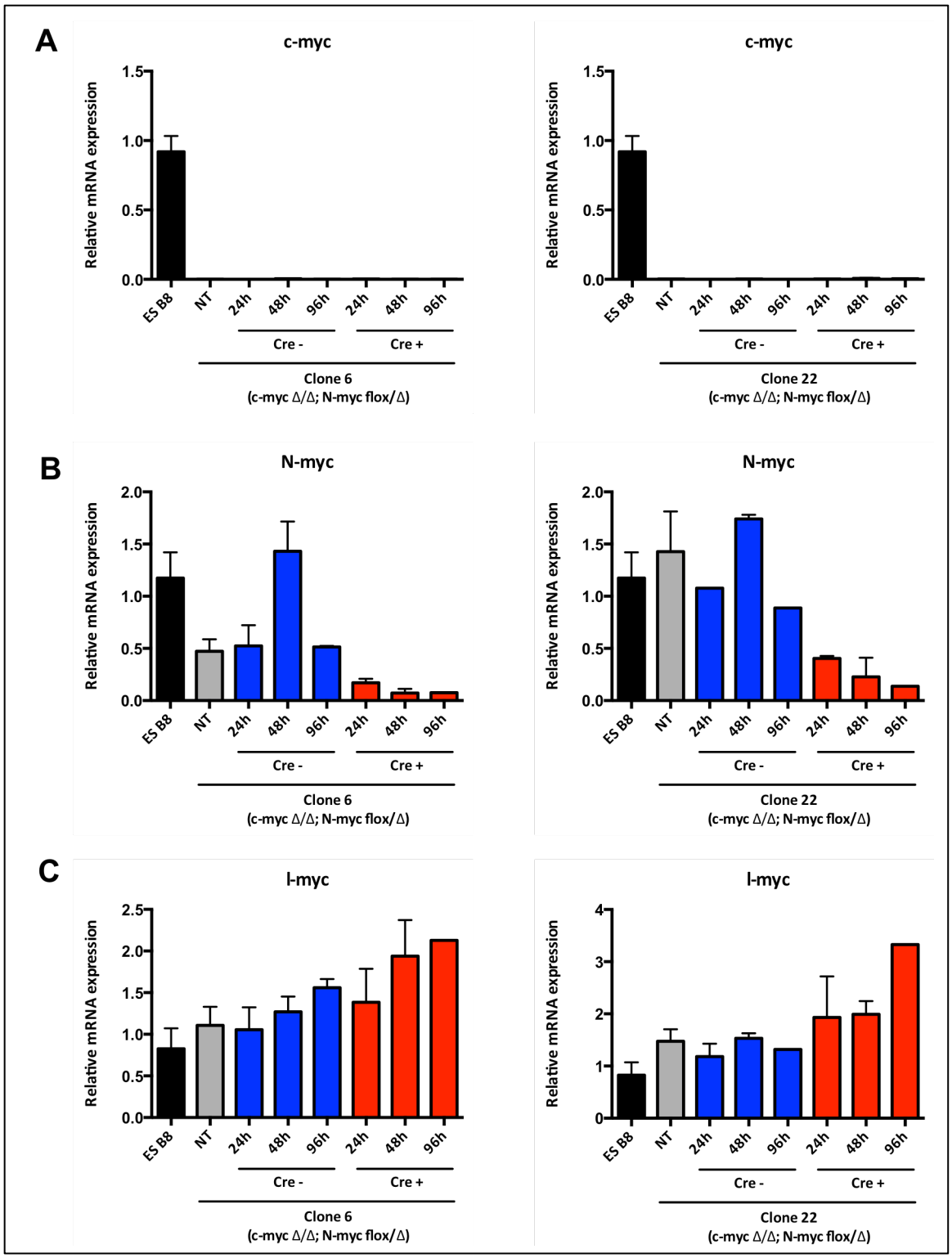

Figure 17: mRNA expression of Myc homologues in mouse ES cells upon Cre mediated c-myc and N-myc deletion. Clone 6 and Clone 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were transiently transfected with the mCherry-Cre plasmid. 24 hours after transfection, Cre+ and Cre- cells were FACS sorted and plated in 12-well plates at a density of 5x10^4 cells per well. mRNA expression levels of c-myc (**A**), N-myc (**B**) and L-myc (**C**) relative to GAPDH were measured by qRT-PCR 24, 48 and 96 hours after transfection (N=3; mean and SEM). ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$) control. NT (not tranfected) control.

The fluorochrome mCherry was used as a reporter of Cre expression (Figure 16.A). 24 hours after transfection, mCherry positive (Cre+) and mCherry negative (Cre-) cells were FACS sorted and immediately used for DNA isolation. Genotyping PCR confirmed the genetic deletion of the last N-myc allele in the Cre+ populations (Figure 16.B).

To confirm that the genetic deletion of myc resulted in the down-regulation of the myc mRNA transcripts, we transfected clone 6 and clone 22 with the mCherry-Cre plasmid and FACS sorted the cells 24 hours after transfection. After sort, Cre+ and Cre- cells were further cultured in 2i + LIF medium. RNA was isolated 24, 48 or 96 hours after Cre induction. As shown in Figure 17.A, qRT-PCR confirmed the absence of c-myc transcripts in clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$). Compared to the c-myc$^{flox/flox}$; N-myc$^{flox/flox}$ control (B8), in the Cre+ cells N-myc expression was strongly reduced already 24 hours after transfection and it further decreased after 48 and 96 hours (Figure 17.B). As a compensatory effect, c-myc and N-myc loss also resulted in a 2 to 3 fold up-regulation of the third Myc family member, L-myc, 96 hours after transfection (Figure 17.C).

5.1.4 Differentiation potential of c-myc KO ES cells

To test the differentiation potential of ES cells lacking both c-myc alleles, we injected c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$ ES cells subcutaneously into immunodeficient NSG mice. Interestingly, although loss of either c-myc or N-myc has been reported to be embryonically lethal due to developmental defects (Charron et al., 1992; Davis et al., 1993; Sawai et al., 1991; Trumpp et al., 2001), we observe that c-myc null ES cells retain the capacity to differentiate into tissues derived from all the three germ layers (Figure 18).

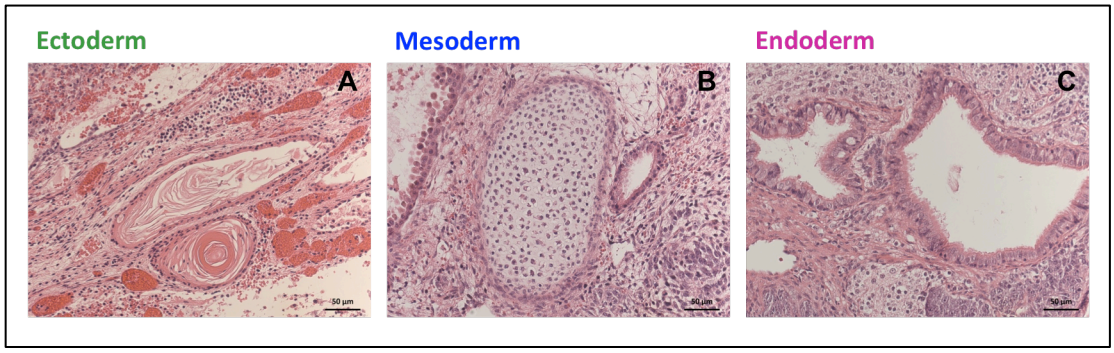

Figure 18: Histology of teratomas derived from c-myc$^{\Delta/\Delta}$, N-myc$^{\Delta/flox}$ ES cells. H&E staining of representative structures of ectodermal (**A**), mesodermal (**B**) and endodermal (**C**) origin derived from teratomas developed from the Clone 4 (c-mycΔ/Δ; N-mycΔ/flox). Scale bars 50µm.

5.1.5 Cell cycle profile of Myc dKO ES cells

Mouse ES cells proliferate very rapidly, with a division time of around 8-10 hours (Stead et al., 2002). Indeed, while somatic cells spend the majority of their time in G1 phase, mouse ES cells are characterized by truncated gap phases, accounting for their rapid cell cycle. Myc proteins are known to have a unique and crucial role in promoting cell proliferation (Alevizopoulos et al., 1997; Daksis et al., 1994; Vlach et al., 1996). Indeed, Myc has been shown to induce cell cycle progression through a variety of mechanisms, as activation of cyclins, inhibition cyclin-dependent kinase (CDK) inhibitors and inhibition of cell cycle checkpoint genes (reviewed in (Meyer and Penn, 2008)). In mouse ES cells cultured in serum + LIF, lack of Myc disrupts their self-renewal capacity, resulting in cell cycle arrest (Smith et al., 2010; Varlakhanova et al., 2010).

To determine the effect of Myc deletion in naïve ES cells, we performed cell cycle analysis combining Ki67 and Hoechst staining on c-myc$^{flox/flox}$; N-myc$^{flox/flox}$ (B8) cells, c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$ (clone 6 and clone 22) cells, and Myc dKO ES cells 96 hours after Cre transfection (Figure 19.A and B).

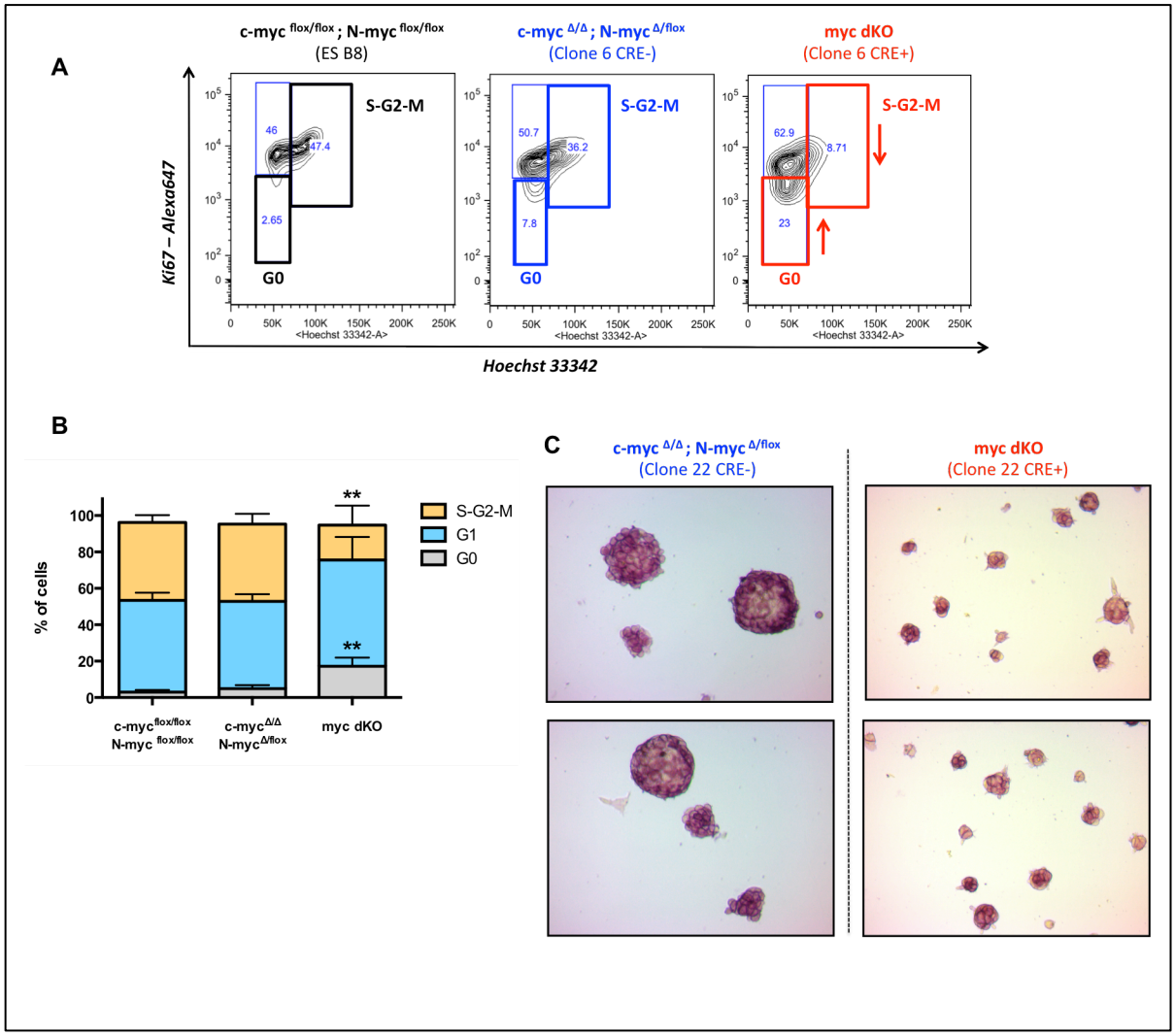

Figure 19: Cell cycle analysis of mouse ES cells upon c-Myc and N-Myc deletion. Clone 6 and Clone 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were transiently transfected with the mCherry-Cre plasmid. 24 hours after transfection, Cre+ and Cre- cells were FACS sorted and plated in 12-well plates at a density of 5x10^4 cells per well. Cell cycle analysis (**A,B**) or alkaline phosphatase (AP) staining (**C**) were performed 72 h after sort.

Exclusive deletion of c-myc seems to have no influence on the proliferative status of mouse ES cells (clones 6 and 22) as a consequence of a possible compensatory role of N-myc (Figure 19.A and B). In contrast, in Myc dKO cells (Cre+) the cell cycle is dramatically affected, resulting in the accumulation of the cells in G0 phase and the

corresponding decrease of proliferative cells in S-G2-M phases (Figure 19.A and B). In agreement with this observation, dKO ES cell colonies are smaller in size as observed by microscopy (Figure 19.C). In conclusion, our results indicate that c- and N-myc loss impairs cell proliferation and this could explain the failure to propagate dKO ES cells in culture.

5.1.6 Pluripotency of Myc dKO ES cells

When ES cells differentiate, the length of the G1 phase increases (White and Dalton, 2005; White et al., 2005) and the rate of cell division is reduced. Along this line, Smith and colleagues observed that, when cultured in serum + LIF medium, Myc dKO ES cells arrest proliferation and rapidly undergo spontaneous differentiation towards primitive endoderm (Smith et al., 2010).

Therefore we tested if the cell cycle arrest caused by loss of myc is also associated to the inactivation of the pluripotency network in the naïve state. Interestingly, 96 hours after Cre induction we observe that in 2i + LIF medium Myc dKO ES cells form smaller, undifferentiated colonies and express the stem cells marker alkaline-phosphates (Figure 19.C). More importantly, Myc dKO ES cells retain protein expression of the pluripotency master regulator Nanog (Figure 20.A and B) as measured by immunofluorescence and FACS analysis, and express the core stem cell factors Oct4 and Sox2 at levels similar to the parental lines (Figure 20.B).

Our results indicate that c- and N-myc loss in ES cells impairs proliferation but it does not impact in the expression of the core pluripotency factors.

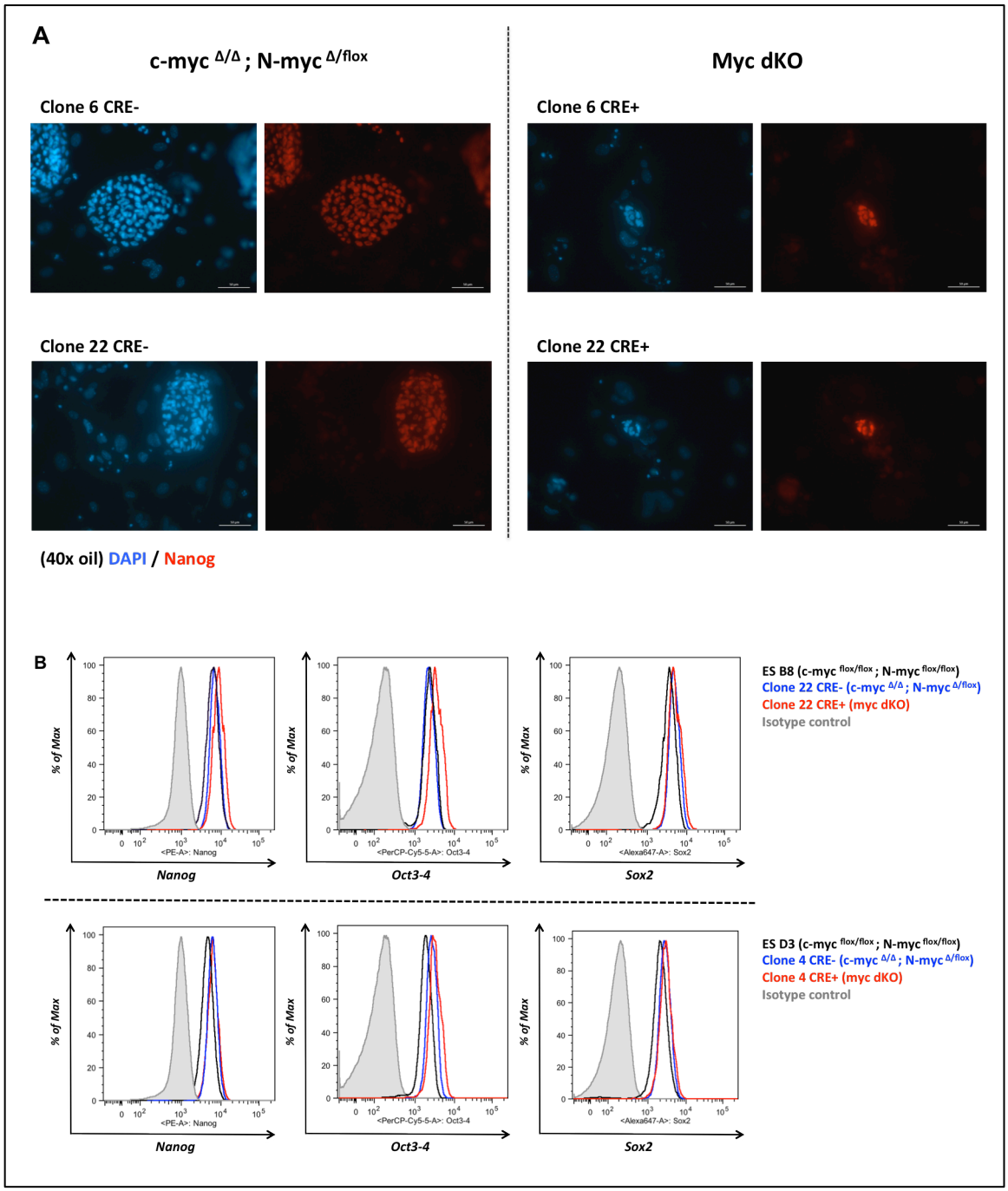

Figure 20: Expression of pluripotency markers in Myc dKO ES cells. Clones 4, 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were transfected with the mCherry-Cre plasmid and Cre+ and Cre- cells were sorted 24 hours after Cre induction. For immunofluorescence staining of Nanog (**A**) 5x10^3 cells were plated on chambered cover glasses coated with MEFs and stained 96 hours after transfection. For intracellular FACS analysis of Nanog, Sox2 and Oct4 (**B**) 5x10^4 sorted cells were plated on gelatin-coated 12-well plates and harvested 96 hours after transfection.

5.1.7 Expression of DNA methyltransferases in Myc dKO cells

In somatic cells, during each round of DNA replication, Dnmt1, the so-called maintenance methyltransferase, propagates the methylation pattern of the mother cell to the daughters, by copying the DNA methylation information from the parental strand of DNA to the newly synthesized strand (Bock and Wutz, 2013). The *de novo* methyltransferases, Dnmt3a and Dnmt3b, are highly expressed in ES cells cultured in serum. In contrast, in 2i conditions, where ES cells more closely resemble the early mouse embryo, the transcription factor Prdm14 down-regulates the expression of the *de novo* methyltransferases leading to globlal DNA hypomethylation (Grabole et al., 2013; Leitch et al., 2013; Yamaji et al., 2013).

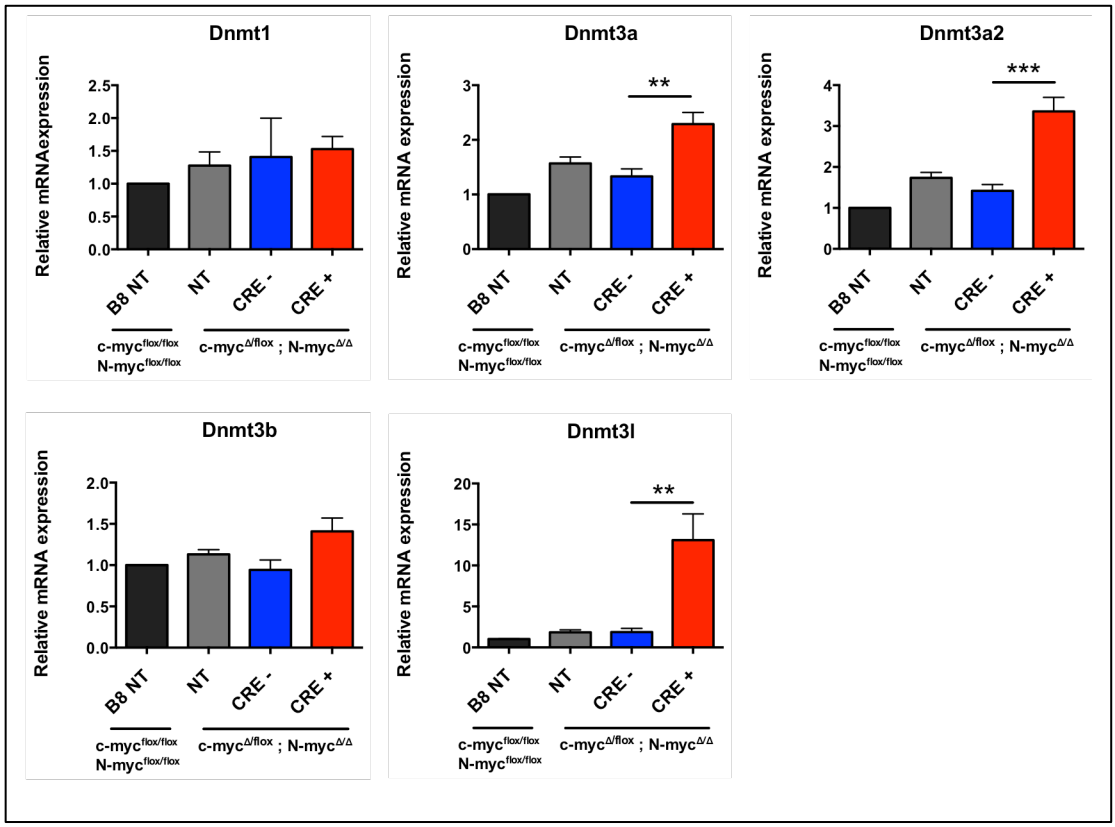

Figure 21: Expression of Dnmts in Myc dKO ES cells. Clone 6 and Clone 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) were transiently transfected with mCherry-Cre plasmid. 24 hours after transfection, Cre+ and Cre- cells were FACS sorted and plated in 12-well plates at a density of 5x10^4 cells per well. 96 hours after transfection, mRNA expression levels of Dnmt1, Dnmt3a, Dnmt3a2, Dnmt3b and Dnmt3l relative to Gapdh were measured by qRT-PCR (N=5; mean and SEM). NT (not tranfected) control.

Considering the importance of these methyltransferases in maintaining the methylation status of naïve ES cells, we measured their mRNA expression levels in Myc dKO cells 96 hours after Cre induction. We found that in Myc dKO cells the levels of the maintenance methyltransferase Dnmt1 and the *de novo* methyltransferase Dnmt3b were unchanged (Figure 21) while we observed a small increase in the mRNA expression of Dnmt3a and its isoform Dnmt3a2. Strikingly, we find that Dnmt3l, a member of the Dnmt family lacking enzymatic activity, is strongly induced in Myc dKO ES cells.

5.1.8 Global gene expression profiling of Myc dKO ES cells

To determine the molecular landscape of Myc dKO ES cells, we performed whole transcriptome analysis (RNA-seq) 24 and 96 hours after Cre induction.

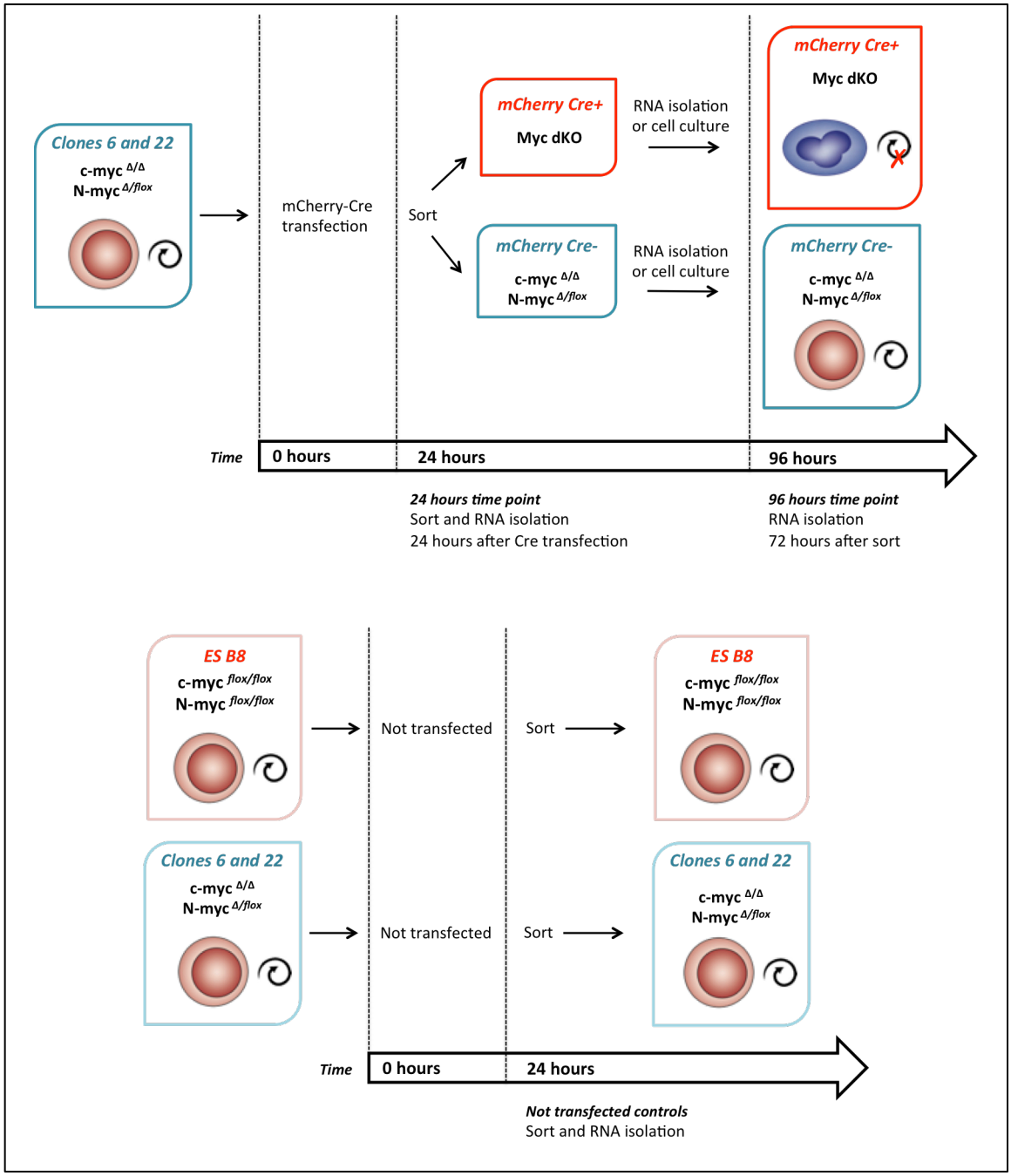

Figure 22: Experimental scheme for RNA-seq of myc dKO ES cells. (**A**) Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted and used for RNA isolation (24 hours time point), or were plated in 24-well plates at a density of 5x10^4 cells/well, in 2i + LIF medium. Cultured cells were harvested 72 hours after sort and used for RNA-isolation (96 hours time point). (**B**) B8 cells (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$) and clones 6 and 22 not transfected, were sorted and used as additional controls.

These two time-points were chosen to distinguish the early and the late events occurring upon loss of myc. The experimental scheme used is illustrated in Figure 22.

Validation of the RNA-seq data

In order to validate our RNA-seq data, we checked the expression levels of the three members of the Myc family (Figure 23). As expected, we found that the levels of c-myc transcripts were significantly lower (DESeq2; p adjusted value < 0.1) in all the c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$ samples where c-myc was genetically deleted. Already 24 hours after Cre induction, N-myc expression was significantly reduced in the Cre+ samples compared to the Cre- and the parental ES B8 line (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$). The third member of the Myc family, L-myc, was slightly although not significantly higher expressed.

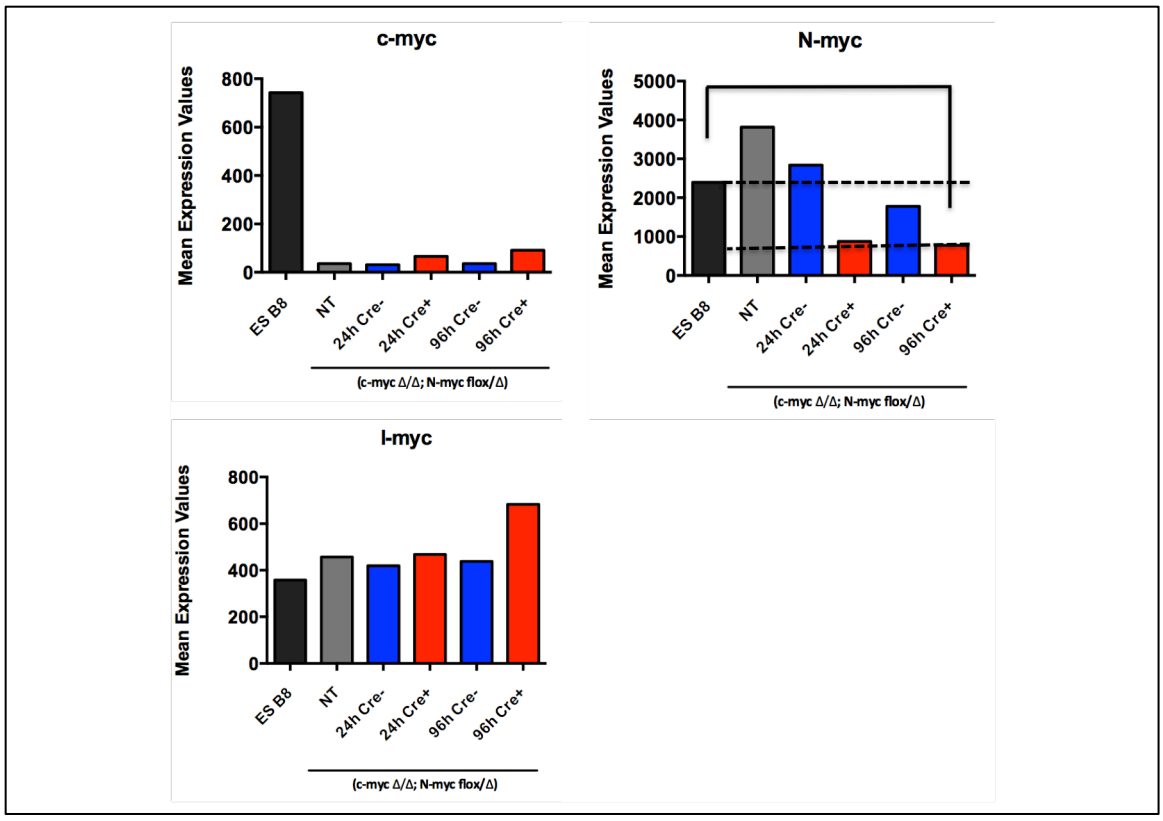

Figure 23: RNA-seq expression levels of the Myc family members. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted. RNA was isolated 24 hours or 96 hours after Cre induction. ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$); NT (not transfected control).

Pluripotency

Consistent with the observation that Myc dKO ES cells maintain protein expression of the core pluripotency factors Oct4, Nanog and Sox2 (Figure 20), RNA-seq analysis shows that 96 hours after Myc deletion, the mRNA levels of these genes remain unchanged (Figure 24).

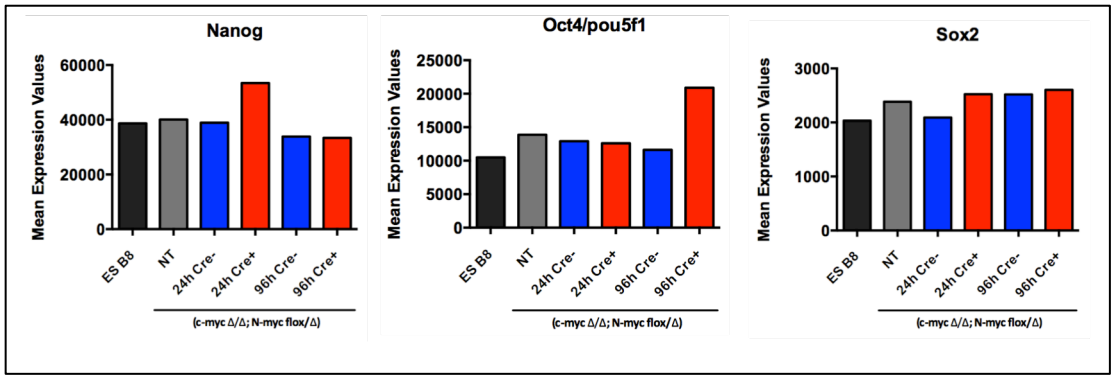

Figure 24: RNA-seq expression levels of the core pluripotency factors Nanog, Oct4 and Sox2. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted. RNA was isolated 24 hours or 96 hours after Cre induction. ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$); NT (not transfected control).

Besides the core pluripotency factors, we extended our analysis to an available published pluripotency signature (Marks et al., 2012) including additional factors known to play a role in ES cells pluripotency.

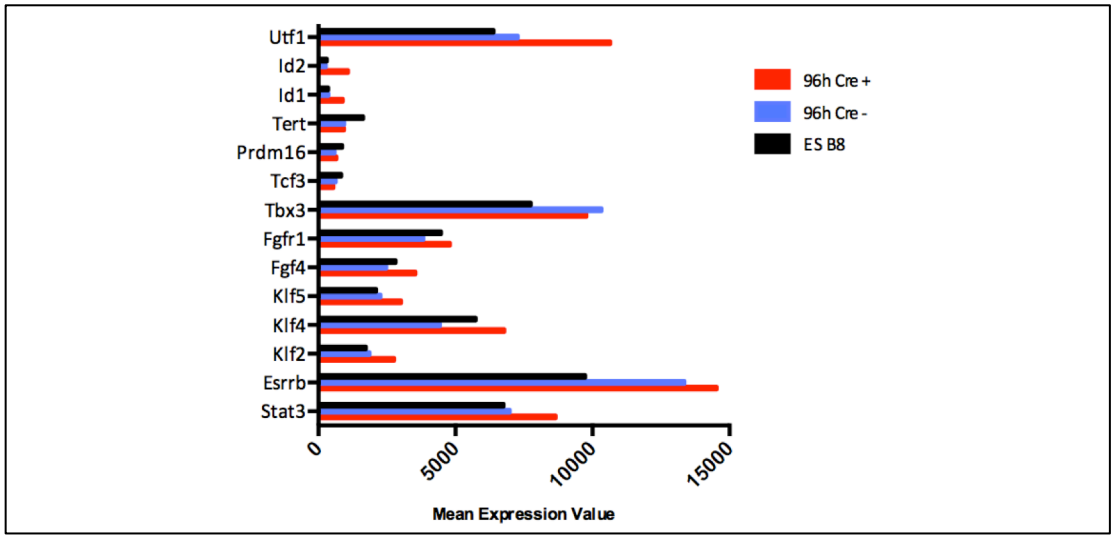

Figure 25: RNA-seq expression levels of relevant pluripotency, self-renewal, and stem cell factors. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted. RNA was isolated 24 hours or 96 hours after Cre induction. ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$); NT (not transfected control).

Our data confirm that several categorized stem cell maintenance genes, as Zfp42 (Rex1), Esrrb, Klf2, Klf4 and Tbx3, are transcribed to similar levels in Myc dKO ES cells compared to the parental cell line (Figure 25). These data are consistent with the hypothesis that upon loss of Myc cell cycle arrest and metabolic quiescence do not impact on the pluripotency network.

Lineage differentiation

When cultured in serum + LIF, Myc dKO mouse ES cells undergo rapid differentiation towards primitive endoderm (Smith et al., 2010). This differentiation program is apparently triggered by the increase expression (40 fold) of the master regulator Gata6 and is confirmed by the strong induction (300 fold) of the endodermal marker Foxa2 (Smith et al., 2010).

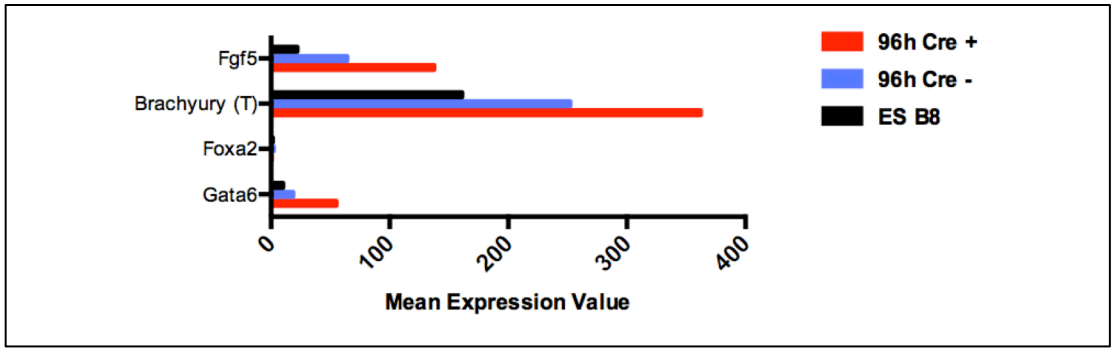

Figure 26: RNA-seq expression of primitive differentiation markers in Myc dKO ES cells grown in 2i + LIF. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted and cultured in 2i + LIF. RNA was isolated 24 hours or 96 hours after Cre induction. ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$).

Interestingly, in the absence of differentiation cues coming from the serum components, we observe only a small, not significant induction of the endoderm markers Gata6 and Foxa2 (Figure 26). The mesodermal marker Brachyury (T) is also slightly but not significantly up-regulated in Myc dKO ES cells, while the primitive ectoderm marker Fgf5, has a 2 fold induction upon loss of both c- and N-Myc (Figure 26).

Gene expression clusters upon Myc deletion

For the analysis of our RNA-seq data, we generated gene expression clusters to identify genes and processes that were specifically up- or down-regulated upon Myc deletion (Figure 27).

Cluster number	ESB8	NT	CRE- 24h	CRE+ 24h	CRE- 96h	CRE+ 96h	# of genes
1	-1	-1	-1	-1	-1	-1	0
2	-1	1	-1	-1	-1	-1	1
3	-1	-1	1	-1	-1	-1	0
4	-1	1	1	-1	-1	-1	19
5	-1	-1	-1	1	-1	-1	2
6	-1	1	-1	1	-1	-1	5
7	-1	-1	1	1	-1	-1	21
8	-1	1	1	1	-1	-1	73
9	-1	-1	-1	-1	1	-1	2
10	-1	1	-1	-1	1	-1	4
11	-1	-1	1	-1	1	-1	3
12	-1	1	1	-1	1	-1	15
13	-1	-1	-1	1	1	-1	22
14	-1	1	-1	1	1	-1	2
15	-1	-1	1	1	1	-1	21
16	-1	1	1	1	1	-1	24
17	-1	-1	-1	-1	-1	1	**70**
18	-1	1	-1	-1	-1	1	79
19	-1	-1	1	-1	-1	1	30
20	-1	1	1	-1	-1	1	86
21	-1	-1	-1	1	-1	1	**93**
22	-1	1	-1	1	-1	1	60
23	-1	-1	1	1	-1	1	116
24	-1	1	1	1	-1	1	75
25	-1	-1	-1	-1	1	1	406
26	-1	1	-1	-1	1	1	100
27	-1	-1	1	-1	1	1	39
28	-1	1	1	-1	1	1	15
29	-1	-1	-1	1	1	1	254
30	-1	1	-1	1	1	1	22
31	-1	-1	1	1	1	1	49
32	-1	1	1	1	1	1	2
33	1	-1	-1	-1	-1	-1	7
34	1	1	-1	-1	-1	-1	44
35	1	-1	1	-1	-1	-1	13
36	1	1	1	-1	-1	-1	219
37	1	-1	-1	1	-1	-1	34
38	1	1	-1	1	-1	-1	42
39	1	-1	1	1	-1	-1	109
40	1	1	1	1	-1	-1	230
41	1	-1	-1	-1	1	-1	45
42	1	1	-1	-1	1	-1	49
43	1	-1	1	-1	1	-1	19
44	1	1	1	-1	1	-1	**155**
45	1	-1	-1	1	1	-1	56
46	1	1	-1	1	1	-1	11
47	1	-1	1	1	1	-1	40
48	1	1	1	1	1	-1	**60**
49	1	-1	-1	-1	-1	1	39
50	1	1	-1	-1	-1	1	39
51	1	-1	1	-1	-1	1	15
52	1	1	1	-1	-1	1	18
53	1	-1	-1	1	-1	1	42
54	1	1	-1	1	-1	1	12
55	1	-1	1	1	-1	1	20
56	1	1	1	1	-1	1	6
57	1	-1	-1	-1	1	1	112
58	1	1	-1	-1	1	1	15
59	1	-1	1	-1	1	1	1
60	1	1	1	-1	1	1	0
61	1	-1	-1	1	1	1	26
62	1	1	-1	1	1	1	1
63	1	-1	1	1	1	1	1
64	1	1	1	1	1	1	0

Figure 27: Gene expression clusters of genes up-regulated (dark green) or down-regulated (light green) in the different sample groups. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted and used for RNA isolation (24 hours time point), or were plated in 24-well plates at a density of $5x10^4$ cells/well in 2i + LIF medium. Cultured cells were harvested 72 hours after sort and used for RNA-isolation (96 hours time point) and sequencing (RNA-seq). ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$); NT (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$).

Since we included several controls in the experiment, we found 64 clusters consisting of all different possible combinations of up- and down-regulated genes in the different samples (Figure 27). Using this matrix we could exclude the possible technical biases introduced by the sorting or the culture.

In the following analysis we focused on clusters number 44 and 48, including genes that are down-regulated 24 and 96 hours after Cre induction, and on clusters number 17 and 21, including genes up-regulated 24 and 96 hours after Cre induction. To further understand the overall biological meaning of the differentially expressed genes we applied Gene Ontology (GO) enrichment analysis.

padjust	name	de.inGO
1.0E-04	gene expression	57
1.2E-05	ribonucleoprotein complex biogenesis	14
1.9E-05	ribonucleoside monophosphate biosynthetic process	6
5.9E-05	ribosome biogenesis	12
9.3E-05	RNA metabolic process	50
1.0E-06	organic cyclic compound metabolic process	69
1.0E-06	heterocycle metabolic process	68
2.1E-06	cellular aromatic compound metabolic process	67
2.1E-06	nucleobase-containing compound metabolic process	66
2.3E-06	nitrogen compound metabolic process	70
5.3E-06	cellular nitrogen compound metabolic process	67
9.3E-05	ribonucleoside monophosphate metabolic process	6
9.3E-05	nucleoside monophosphate metabolic process	6
1.0E-04	cellular metabolic process	87
9.9E-12	nucleolus	30
2.0E-10	nuclear part	53
5.2E-08	nucleus	79
7.2E-08	nuclear lumen	42
1.8E-05	nucleobase biosynthetic process	5
1.9E-05	nucleoside monophosphate biosynthetic process	6
7.8E-05	nucleobase metabolic process	5
2.0E-03	purine nucleoside monophosphate biosynthetic process	4
2.9E-03	purine nucleobase biosynthetic process	3
2.9E-03	purine nucleobase metabolic process	3
3.6E-03	pyrimidine-containing compound biosynthetic process	4
3.6E-03	pyrimidine ribonucleoside biosynthetic process	4
3.6E-03	pyrimidine nucleotide biosynthetic process	4
3.6E-03	pyrimidine ribonucleotide biosynthetic process	4
3.6E-03	pyrimidine nucleoside biosynthetic process	4
6.0E-03	purine ribonucleoside monophosphate metabolic process	4
6.0E-03	purine nucleoside monophosphate metabolic process	4
6.0E-03	pyrimidine ribonucleoside metabolic process	4
6.0E-03	pyrimidine ribonucleotide metabolic process	4
6.0E-03	pyrimidine nucleotide metabolic process	4
6.0E-03	pyrimidine nucleoside metabolic process	4
7.1E-04	ncRNA metabolic process	12

Figure 28: Processes down-regulated in Myc dKO ES cells 24 and 96 hours after Cre induction. Table of enriched GOs grouped by process similarity. Ribosome biogenesis (red); gene expression (light blue); metabolism (green); nuclear components (yellow) and nucleotide synthesis (purple). Left column: p adjusted value. Right column: number of genes in the data set functionally related to the process.

Processes down-regulated in Myc dKO ES cells

GO annotation enrichment analysis of the 155 genes belonging to the cluster 44 (transcripts down-regulated upon Myc deletion at 24 and 96 hours) shows a distinct signature in processes related to metabolism as seen by ribosomal biogenesis, RNA and DNA biosynthesis (Figure 28). These data suggest that Myc dKO ES cells enter a state of "metabolic quiescence", characterized by a strong reduction of several essential metabolic activities, including protein and nucleotide synthesis.

Metabolism

When we examine the cellular processes that are altered upon Myc loss, cell metabolism is certainly the most prominent. Specifically, we observe that in the absence of c- and N-Myc activity several core metabolic processes are down-regulated, including ribosomal biogenesis and nucleotide synthesis.

Myc proteins have been described to be involved in multiple aspects of ribosomal biogenesis by regulating the expression of ribosomal proteins and factors required for rRNA processing, ribosome assembly, export of mature ribosomal subunits from the nucleus to the cytoplasm, as well as factors controlling the initiation of mRNA translation (van Riggelen et al., 2010). Consistently, we find that the expression of several genes involved in ribosome biogenesis is reduced following loss of Myc. These genes include nucleolin (**Ncl**), involved in the cleavage of the 47S pre-rRNA into 18S, 5.8S and 28S rRNAs (Ginisty et al., 1998), the ribosome biogenesis protein **Wdr12**, component of the PeBoW complex, required for maturation of 28S and 5.8S ribosomal RNAs and formation of the 60S ribosome (Holzel et al., 2005) and **Rplp0**, encoding a ribosomal protein component of the 60S subunit.

Interestingly, the eukaryotic initiation factor 3a (**Eif3a**), component of the eukaryotic translation initiation factor 3 (eIF-3) complex, is also down-regulated upon Myc deletion. tRNAs are essential intermediates of protein synthesis, since they translate the mRNA ribonucleotide code into a polypeptide sequence. tRNAs synthesized in the nucleus are actively exported to the cytoplasm by specific transport factors. The balance between nuclear and cytoplasmic pools of tRNA is regulated by nutrient

availability, with starvation causing a rapid and reversible accumulation of tRNA in the nucleus (Neufeld and Arsham, 2010). Interestingly, in our RNA-seq analysis we find that exportin-T (**Xpot**), mediating the nuclear export of aminoacylated tRNAs (Kutay et al., 1998), is also downregulated in dKO ES cells.

Following Myc deletion, a number of genes involved in nucleotide biosynthesis are also repressed: **Gart**, is a tri-functional purine biosynthetic protein adenosine-3 required for *de novo* purine biosynthesis (Kan et al., 1993); **Atic**, encodes for the bi-functional purine biosynthesis protein Purh; **Aprt**, is an adenine phosphoribosyltransferase, also involved in purine biosynthesis. The uridine-cytidine kinase 2 (**Uck2**) is the mouse homologue of the human Uck2 gene, which phosphorylates uridine and cytidine to uridine monophosphate (UMP) and cytidine monophosphate (CMP) (Suzuki et al., 2004). Uck2, involved in pyrimidine ribonucleoside biosynthesis, is also down-regulated upon Myc loss.

Cell cycle

In agreement with the observation that Myc loss induces cell cycle arrest, global gene expression profiling of Myc dKO ES cells shows that genes driving cell cycle progression are significantly down-regulated following Myc loss (Figure 29). This group of genes includes **E2f3**, member of the E2F family of transcription factors, activating genes essential for DNA replication (Helin, 1998) and known to be critical for normal cellular proliferation (Humbert et al., 2000). **Skp2**, encoding the S-phase kinase-associated protein 2 and **Hmga1**, encoding the mobility group AT-hook 1 protein, are other examples of genes driving cellular proliferation and repressed in myc dKO ES cells. Along the same line, well-known repressors of the cell cycle as **Gadd45g**, a member of the growth arrest and DNA damage-inducible protein family, and **Cdkn1c**, encoding the cell cycle inhibitor p57, are strongly induced 96 hours after myc deletion. Taken together these results indicate that ES cells strictly depend on Myc expression to undergo cell division, and this effect is independent on the culture conditions.

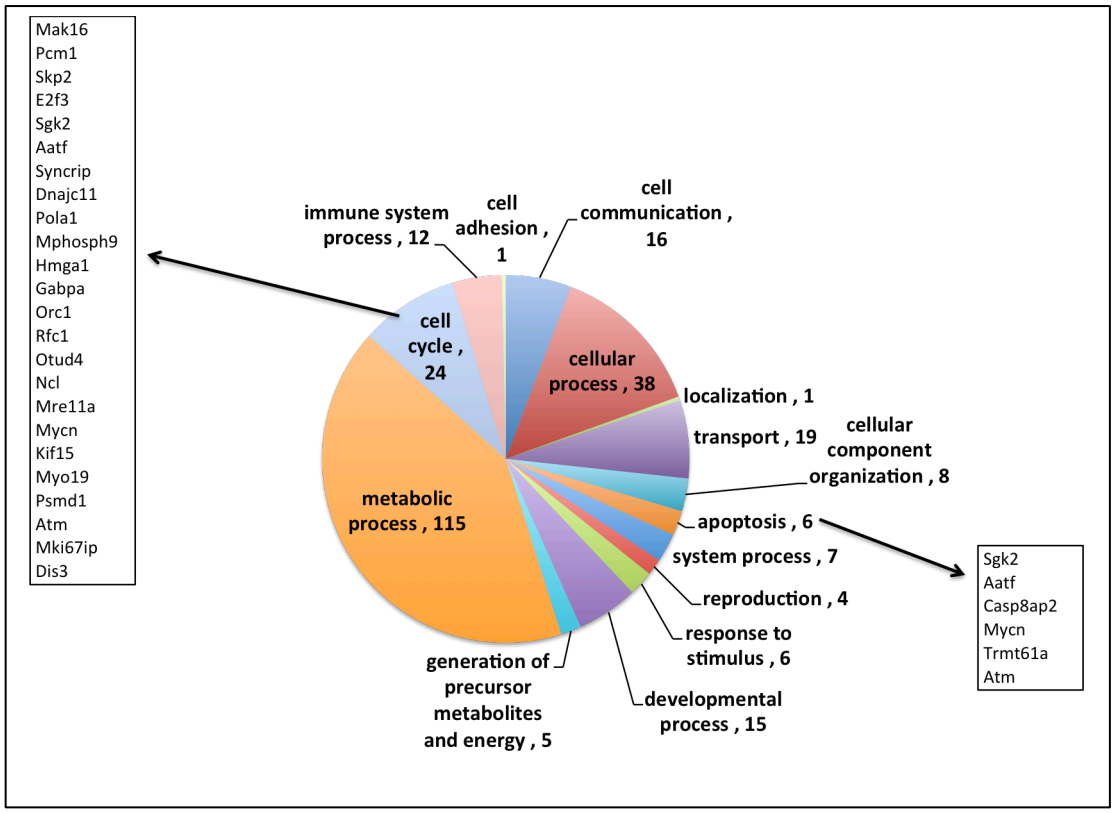

Figure 29: Processes and genes down-regulated in Myc dKO ES cells. Functional annotation clustering of differentially expressed genes by Gene Ontology ([GO] PANTHER [protein analysis through evolutionary relationships]) analysis.

Processes up-regulated in Myc dKO ES cells

GO annotation enrichment analysis of the 93 genes belonging to the cluster 21 (transcripts up-regulated 96 hours after Cre induction) shows a distinct signature in processes related to insulin signaling (Figure 30).

Fcer1g,Unc93b1,Cd74	0.01268051	antigen processing and presentation of exogenous peptide antigen
Bax,Irs2,Cd74	0.01268051	B cell apoptotic process
Irs2,Igf2,Igf1r	0.02340943	insulin receptor binding
Irs2,Cd74,Hes1,Igf2	0.03071975	positive regulation of mononuclear cell proliferation
Msln,Hes1,Igf2,Igf1r	0.04839653	pancreas development
Cd74,Fcer1g,Unc93b1,Mdfi,Hes1,Rhoc,Igf1r,Igf2,Bax,Clu	0.06476445	positive regulation of response to stimulus
Msln,Prnd,Dpep3	0.08036566	anchored to membrane
Irs2,Igf1r,Igf2	0.08036566	positive regulation of lipid metabolic process
Irs2,Cd74,Hes1,Igf2	0.0883738	regulation of leukocyte proliferation
Cd74,Igf2,Ccnd2,Mdfi,Bax,Irs2,Hes1,Igf1r	0.09643279	regulation of phosphorylation
Cd74,Mdfi,Hes1,Rhoc,Igf1r,Igf2,Bax,Clu	0.09643279	positive regulation of signal transduction
Cd74,Mdfi,Hes1,Rhoc,Igf1r,Igf2	0.09643279	positive regulation of intracellular protein kinase cascade
Bax,Ndn,Nfasc,Klf7,Hes1,Irs2,Igf1r,Fcer1g,Cd74	0.11696676	locomotion
Bax,Irs2,Cd74,Fcer1g,1110007C09Rik,Clu,Tsc22d1,Igf1r	0.12905648	regulation of cell death
Bax,Irs2,Cd74,Fcer1g,1110007C09Rik,Clu,Tsc22d1,Igf1r	0.12905648	regulation of programmed cell death
Irs2,Rhoc,Cd74,Olfr487,Gpr64,Igf1r,Fcer1g	0.12905648	signal transducer activity
Ndn,Prnd,Bax,Nfasc,Scn10a,Celf4	0.12905648	cellular ion homeostasis
Hes1,Igf1r,Ndn,Klf7,Nfasc	0.12905648	cell morphogenesis involved in neuron differentiation

Figure 30: Processes up-regulated in Myc dKO ES cells 96 hours after Cre induction. Table of enriched GOs and genes functionally related to the process. P adjusted values are shown in green.

Upon Myc deletion the insulin-growth-factor-1 receptor **Igf1r** and the insulin receptor substrate proteins **Irs2** and **Irs3**, are induced 96 hours after Cre induction (Figure 31). Indeed, insulin signaling is one of the most significantly represented up-regulated processes (Figure 30). Interestingly, the Forkhead box protein O3 (**Foxo3**) is also induced upon loss of Myc.

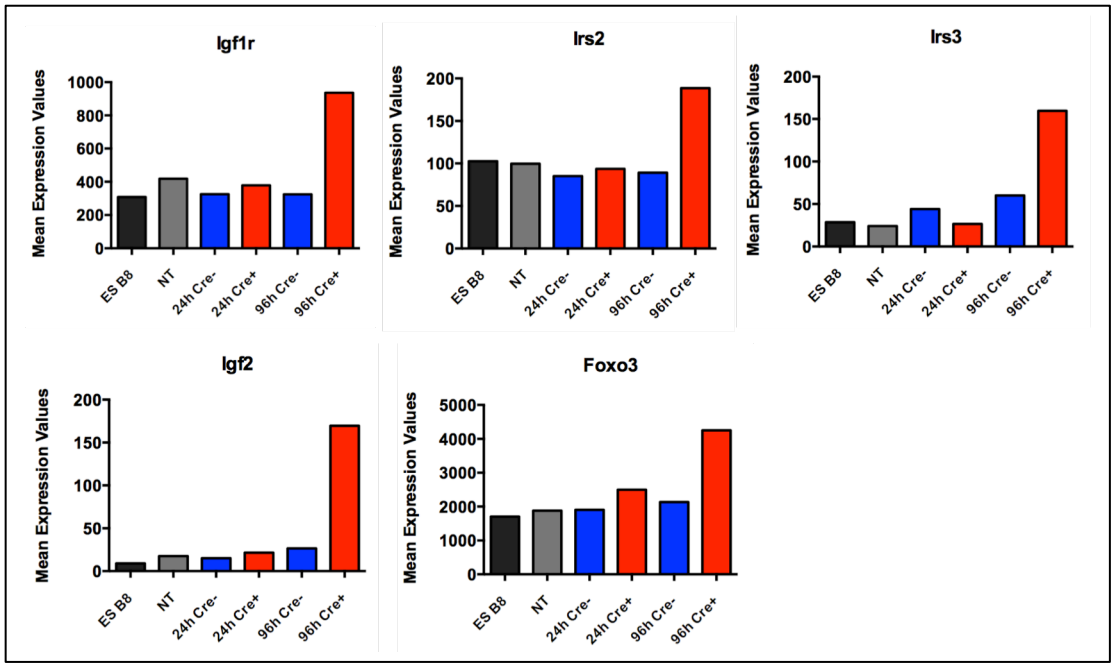

Figure 31: RNA-seq expression levels of factors involved in the insulin-signaling pathway. Clones 6 and 22 (c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$) cultured in 2i + LIF medium were transfected with the mCherry-Cre plasmid. 24 hours after transfection, mCherry+ (Cre+) and mCherry- (Cre-) cells were FACS sorted. RNA was isolated 24 hours or 96 hours after Cre induction. ES B8 (c-myc$^{flox/flox}$; N-myc$^{flox/flox}$); NT (not transfected control).

5.2 Comparison of ES cells expressing different c-Myc protein levels

5.2.1 c-Myc expression in 2i + LIF and in serum + LIF

RNA-seq data from the group of Austin Smith have reported that c-myc mRNA level is 40 to 50 fold lower in 2i than in serum, and N-myc and L-myc expressions are also reduced in 2i (Marks et al., 2012).

In order to quantify c-Myc protein expression in the different culture conditions, we made use of the e-GFP-cMyc knock-in mice generated and characterized by the group of Berry Sleckman (Huang et al., 2008). These mice are carrying a c-Myc^{GFP} allele, encoding for a GFP-cMyc fusion protein from the endogenous *c-myc* locus.

Mice homozygous for c-Myc^{GFP} are viable, appear normal and have no alterations in the kinetics of hematopoietic differentiation or immune function (Huang et al., 2008; Reavie et al., 2010), suggesting that the N-terminal fusion of GFP to c-Myc does not affect c-Myc function. This N-terminally tagged c-Myc protein can be therefore used as reporter of c-Myc protein expression.

In collaboration with the transgenic service of the DKFZ, we derived 6 homozygous c-Myc$^{GFP/GFP}$ mouse ES cell lines. These lines have been cultured in 2i + LIF medium for several passages, and have been adapted to grow in feeder-free conditions, on 0.1% gelatin, where they form undifferentiated colonies. As reported for several other mouse ES cell lines, in serum-containing medium the c-Myc$^{GFP/GFP}$ ES cells are not able to sustain their growth in the absence of MEFs and rapidly differentiate. Therefore, to compare c-Myc protein expression in the different culture conditions, we cultured c-Myc$^{GFP/GFP}$ ES cells on a feeder layer of MEF constitutively expressing a membrane-targeted tdTomato fluorescent protein and grew the cells for 72 hours in 2i + LIF or in serum + LIF medium (Figure 32). We than performed FACS analysis to quantify c-Myc-GFP levels and used as a control for auto-fluorescence E14 ES cells, derived from 129/Ola-Hsd mice, not expressing the GFP fluorochrome. Our data confirm that c-Myc-GFP protein expression is very low in culture conditions supporting the naïve state and it strongly increases when ES cells are transferred from 2i to serum (Figure 32). From these results we can hypothesize that c-myc is one of the early genes up-regulated upon exit from the naïve state of pluripotency.

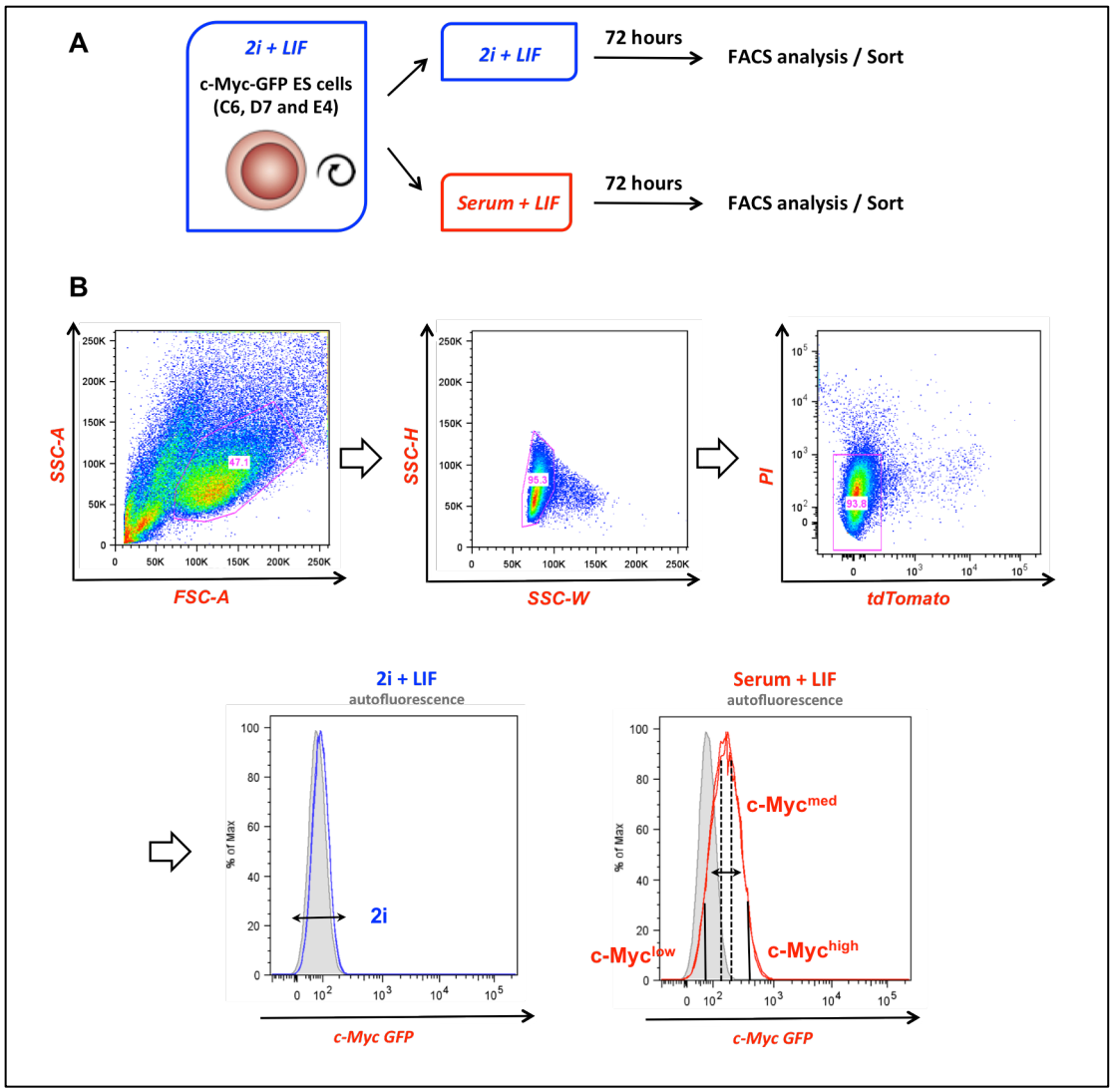

Figure 32: Expression levels of c-Myc-GFP protein in 2i + LIF and in serum + LIF medium. (**A**) The c-Myc$^{GFP/GFP}$ ES cell lines C6, D7 and E4 were culture in 2i + LIF medium or serum + LIF medium for 72 hours on tdTomato MEFs. (**B**) c-Myc protein levels were quantified by FACS analysis gating on PI negative, tdTomato negative cells. E14 ES cells were used as a control for auto-fluorescence (in grey).

5.2.2 Properties of ES cells expressing different c-Myc-GFP protein levels in serum + LIF

In order to define the properties of ES cells cultured in serum + LIF and expressing different c-Myc protein levels, we FACS sorted cells according to the levels of c-Myc-GFP expression as showed in Figure 32. We distinguished three populations expressing high, medium and low levels of c-Myc-GFP and analyzed their properties in terms of cell size, RNA-content, and cell cycle profiles.

Two recent studies from the groups of Richard Young and David Levens (Lin et al., 2012; Nie et al., 2012) have shown that c-Myc is a universal amplifier of already active gene expression programs and cells with high levels of c-Myc are bigger in size and produce two to three times more total RNA compared to Myc low cells. According to the published data, we observe that in serum + LIF, c-Myc^{high} cells have almost twice total RNA content compared to c-Myc^{low} cells (Figure 33.A) and are bigger in size (Figure 33.B) as shown by an higher forward scatter (FSC-A).

When we looked at the cell cycle profiles, we observed that c-Myc protein levels correlate with the cell cycle state of the cells (Figure 32.C and D), with the c-Myc^{high} population being mostly in S-G2-M phases and the c-Myc^{low} cells being in G1 phase of the cell cycle.

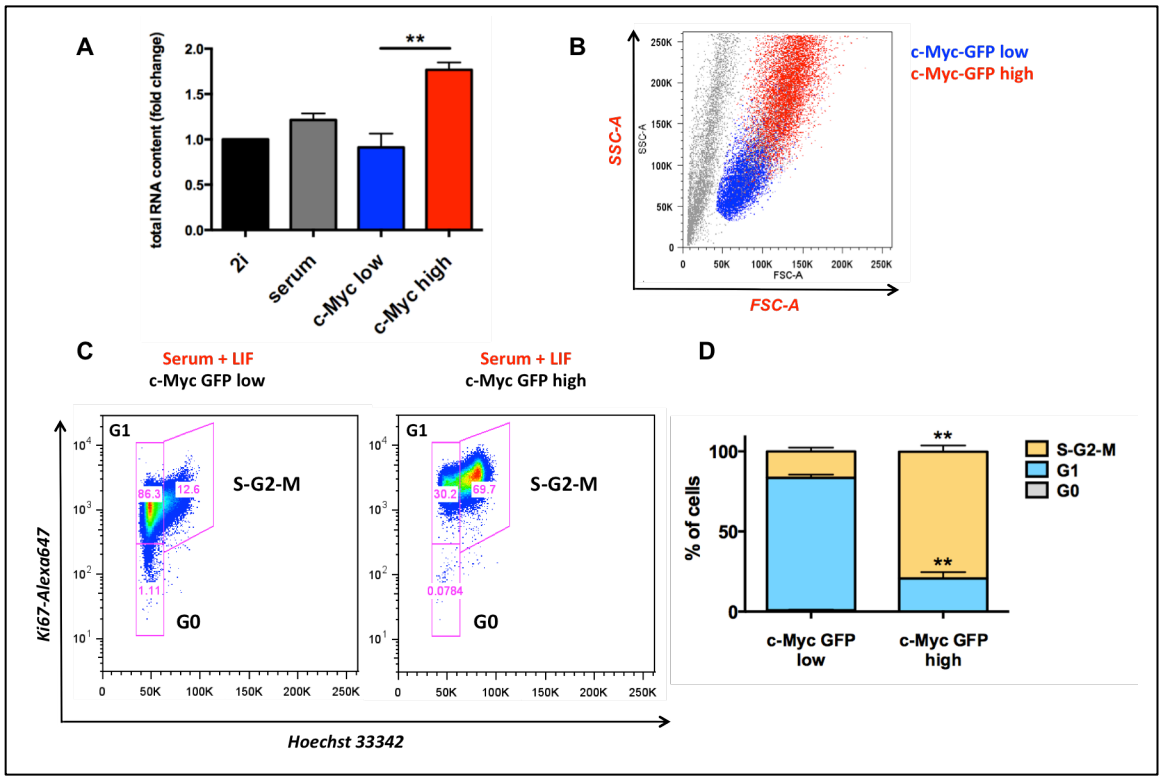

Figure 33: Properties of ES cells expressing different c-Myc protein levels in serum + LIF medium. c-Myc$^{GFP/GFP}$ ES cells were cultured for 72 hours in serum + LIF medium on a layer of tdTomato MEFs. The cells were than FACS sorted according to the levels of c-Myc-GFP expression as shown in **Figure 32**. For quantification of the total RNA content (**A**), 2x10^4 cells from each population were FACS sorted directly in extraction buffer for RNA isolation. To evaluate size differences (**B**), FCS and SSC parameters were assessed by FACS analysis. Ki67-Hoechst staining was used to profile the cell cycle of c-Myc-GFP^{high} and c-Myc-GFP^{low} populations (**C,D**). SSC-A (side-scatter); FSC-A (forward-scatter).

6. Discussion

Pluripotency in the early embryo is defined as the capacity of a single cell to generate all lineages of the adult organism (Young, 2011). *In vivo* this property is possessed only transiently by the cells of the epiblast but it can be indefinitely "captured" *in vitro* by deriving embryonic stem (ES) cells. A second important property of ES cells is self-renewal, defined as the ability of a stem cell to proliferate and generate one or two daughter stem cells. This process requires the coordination of cell-cycle progression and cell fate choices (Orford and Scadden, 2008).

ES cell lines have been generated both from mouse embryos (Evans and Kaufman, 1981; Martin, 1981) and from humans (Thomson et al., 1998). Although a tissue culture source of human pluripotent cells holds a great potential for applications in basic research and transplantation therapies, research on human ES cells generates ethical issues associated to the use of human embryos (Miller and Bloom, 1998). In 2006, the Nobel laureate Shinya Yamanaka observed that overexpression of four transcription factors, Oct4, Sox2, Klf4 and c-Myc, is sufficient to "reprogram" adult mouse cells into induced pluripotent stem (iPS) cells, closely resembling ES cells (Takahashi and Yamanaka, 2006). The four Yamanaka factors were later shown to be able to reprogram also adult human dermal fibroblasts into iPS cells (Takahashi et al., 2007), therefore it is now possible to derive human pluripotent cells in tissue culture not only from limited and ethically challenged human embryos, but also from adult cells (Evans, 2011).
ES cells and iPS cells hold great promise for regenerative medicine, and our understanding of the biology of these stem cells is important both to elucidate the basis of embryonic development and to improve our ability to *in vitro* manipulate stem cells for cell replacement therapy.

When ES cells were cultured for the first time, they were expanded on mitotically inactivated fibroblasts, in a culture medium containing fetal bovine serum (Evans and Kaufman, 1981). Since the serum components represent a source of undefined

signals that could potentially promote differentiation, ES cells cultured in serum-containing medium have been shown to be heterogeneous in morphology and expression of pluripotency markers as Nanog, Stella and Oct4 (Chambers et al., 2007; Hayashi et al., 2008; Marks et al., 2012; Nichols and Smith, 2009, 2012). Culturing ES cells in serum-free medium and in the presence of two inhibitors (2i) of the MAPK and GSK3b pathways is sufficient to capture ES cells in a naïve, ground state of pluripotency, which more closely resembles the stem cell status in the inner cell mass of the blastocyst (Ying et al., 2008). Since different culture environments impose distinctive transcriptional and epigenetic properties on mouse ES cells (Marks et al., 2012), it appears to be necessary to re-evaluate the function of several regulators of pluripotency under 2i conditions.

The effect of the simultaneous c- and N-myc deletion in ES cells cultured in serum + LIF has been recently described (Smith et al., 2010; Varlakhanova et al., 2010). Under these culture conditions, Myc contributes to self-renewal and maintenance of the pluripotent state by repressing the endoderm master regulator Gata6 and regulating the mir-17-92 miRNA cluster (Smith et al., 2010). Therefore, loss of both c- and N-myc alleles in serum + LIF results in cell cycle arrest and spontaneous, rapid differentiation towards primitive endoderm. We know from recent studies that both feeders and serum are sources of undefined signals, driving mouse ES cells towards metastability, therefore it cannot be excluded that the effects of Myc loss observed in serum could be the results of differentiation cues normally absent *in vivo* during early embryogenesis.

The aim of this thesis was to define the role Myc family members in the maintenance of pluripotency and self-renewal of naïve mouse ES cells. To compare the levels of c-Myc protein in naïve and metastable ES cells, we derived ES cell lines from mice in which a GFP gene was introduced in-frame into the endogenous *c-myc* locus to encode for a c-Myc-GFP reporter protein (Huang et al., 2008). Myc proteins are known to be responsive to serum mitogens (Perna et al., 2012) and FACS analysis revealed that while c-Myc protein expression is extremely low in naïve ES cells cultured in 2i + LIF, c-Myc is strongly induced upon withdrawal of the two inhibitors

and addition of serum. This observation suggests that c-Myc expression is induced *in vitro* during the earliest differentiation steps of naïve ES cells. Interestingly, when we analyze ES cell that have been cultured 72 hours in serum + LIF medium, we observe that c-Myc-GFP high cells are bigger in size and have an RNA content 1.5 – 2 fold higher than cells expressing low levels of Myc. Additionally, ES cells expressing high c-Myc levels are mostly in S-G2-M phase of the cell cycle, while c-Myc low cells are mainly in G1 phase.

To genetically address the role of Myc proteins in the naïve state of pluripotency, we derived ES cells where both c-myc and N-myc could be conditionally deleted to generate Myc dKO ES cells (Knoepfler et al., 2002; Trumpp et al., 2001). An important aspect to take into consideration in this study is the observation that Myc family members could have redundant functional properties during the early stages of embryogenesis. Indeed, knockout of individual Myc genes is only lethal at embryonic day 10 or 12, as a consequence of respectively c-myc or N-myc loss (Charron et al., 1992; Davis et al., 1993; Stanton et al., 1992). Consistent with the notion of functional redundancy between c- and N-myc, we find that c-myc$^{\Delta/\Delta}$; N-myc$^{\Delta/flox}$ ES cells can be extensively expanded *in vitro* in 2i + LIF medium, and differentiate into teratomas when subcutaneously injected into immunodeficient mice. While naïve ES cells expressing one N-myc allele show no obvious phenotype, ES cells lacking both myc genes (all four alleles) undergo rapid cell cycle arrest. These data suggests that mouse ES cell proliferation strictly depends on Myc expression.

Myc-dKO ESCs form smaller and arrested colonies, but unexpectedly remain undifferentiated, maintaining normal expression of alkaline phosphatase and, more importantly, of the core pluripotency factors Oct4, Nanog, and Sox2, both at the RNA and protein level. To determine the molecular properties of naive Myc dKO ES cells, we performed whole transcriptome analyses by next-generation sequencing (RNA-seq) 24 and 96 hours after Cre induction. As expected, the data confirmed the unchanged expression signature of the core and expanded pluripotency network.

Global transcriptome analysis of Myc dKO ES cells also revealed that virtually all the metabolic and biosynthetic aspects of cellular physiology were robustly down-regulated in dKO ES cells. These include cell cycle activity, DNA replication, ribosomal biogenesis and DNA and protein synthesis. Our group has recently described the role of Myc proteins in hematopoietic stem cells (HSCs) (Laurenti et al., 2008). Interestingly, deletion of both c- and N-myc in HSCs results in global reduction of ribosome biogenesis, similarly to what we observe in ES cells. Nevertheless, the main effect of Myc loss in the hematopoietic system is a strong apoptotic response, driven by the serine protease Granzyme B (GzmB), resulting in a rapid and severe bone marrow failure (Laurenti et al., 2008). In ES cells, we do not observe any obvious increase in apoptosis, and looking at their gene expression profile, Myc dKO ES cells do not activate any apoptotic program 96 hours after Cre induction. All together our data indicate that the overall cellular and molecular phenotype of Myc-dKO ES cells is consistent with a status of “metabolic and proliferative dormancy”.

The concept of dormancy is not new to the stem cell field: recent studies have shown that in mammals several adult tissues contain a subset of tissue-specific stem cells that persist in a quiescent state for prolonged periods of time and proliferate in response to tissue damage or to compensate for tissue loss. Hence stem cell quiescence is a poised state, actively maintained by specific signaling pathways and quiescent stem cells have the extraordinary capacity to sense environmental changes and are ready for rapid activation (Cheung and Rando, 2013). In the adult mouse, for example, hematopoietic stem cells (HSCs) have the potential to generate the entire hematopoietic system. However, during homeostasis, the population of HSCs harboring the highest self-renewal potential is actually slowly cycling and present in a dormant state (Foudi et al., 2009; Wilson et al., 2008). During homeostasis signals within the bone marrow niche contribute to maintaining HSCs in a metabolically inactive state, while in emergency situations, in response to stress and injury, HSCs exit their quiescent state and proliferate, to efficiently and rapidly reconstitute the hematopoietic system (Wilson et al., 2009). Interestingly, the comparison of gene expression profiles of different types of adult quiescent stem cells including HSCs (Forsberg et al., 2010), muscle stem cells (MuSCs) (Fukada et al.,

2007) and hair follicle stem cells (HFSCs) (Blanpain et al., 2004), shows that all these quiescent stem cells have a common gene signature, the transcriptional landscape of quiescent stem cells (Cheung and Rando, 2013). Consistent with the dormant phenotype, genes that are involved in cell cycle progression, DNA replication or mitochondrial functions are mostly down-regulated in quiescent stem cells (Cheung and Rando, 2013) and in Myc dKO ES cells. Genes up-regulated in quiescent stem cells include genes encoding signaling molecules involved in transcriptional regulation and stem cell fate decisions such as forkhead box O3 (Foxo3). Interestingly, Myc dKO ES cells show an increased expression of the CDK inhibitor p57, described to regulate dormancy of both HSCs and neural stem cells (NSCs) (Furutachi et al., 2013; Matsumoto et al., 2011). p57 could therefore function as a common player in regulating stem cells dormancy.

In almost 100 mammalian species, embryonic development can be reversibly arrested at the time of embryo implantation (Renfree and Shaw, 2000). This delayed implantation, termed “diapause”, is a strategy to enhance the reproductive fitness. Indeed, diapause length is regulated by environmental cues, such as light and temperature, to ensure the most optimal conditions for survival of the offspring (Schiesari and O'Connor, 2013). In mice, facultative diapause occurs to delay implantation of early embryos in mothers still feeding a previous litter. During diapause, mouse blastocysts enter a state of metabolic and proliferative quiescence that represent the dormant state of the embryo (Hondo and Stewart, 2005).
Strikingly, the signature of dKO ES cells maintained in 2i medium is remarkably similar to an expression signature previously reported for diapause arrested pre-implantation embryos (Hamatani et al., 2004). Very low expression of processes such as cell division, metabolism, regulation of transcription and translation, is observed in both diapause embryos and Myc dKO ES cells.

The diapause embryo is also characterized by high expression of the insulin pathway, one of the most up-regulated processes in Myc dKO ES cells. Insulin is the most potent anabolic hormone known. It stimulates the cellular uptake of glucose, amino acids and fatty acids, and increases the expression or activity of enzymes that

catalyze glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyze degradation (Saltiel and Kahn, 2001). Insulin is also likely to influence insect diapause (Sim and Denlinger, 2013), suggesting that this signaling pathway has been evolutionary conserved for diapause in both vertebrates and invertebrates. Although the link of insulin pathway to the dormant mouse blastocyst is not yet clear, it possibly maintains the embryo in a poised state that is ready for a rapid re-activation and exit from quiescence following hormonal stimulation.

Based on the similarities between the dormant embryo and Myc dKO ES cells, our data raise the possibility that Myc activity controls the reversible diapause arrest of early embryos. However, this theory has to be further supported with additional experiments to dissect the mechanism linking Myc activity to blastocyst quiescence and re-activation. We know from previous studies that successful implantation results from reciprocal interactions between an implantation-competent blastocyst and a receptive uterus. Ovarian estrogen and progesterone (P4) coordinate cellular and molecular events to produce a favorable uterine environment (Hamatani et al., 2004). Among other factors, the cytokine LIF is crucial for uterine preparation for implantation (Wang and Dey, 2006). In mice, LIF is expressed first in uterine glands on the morning of day 4, and then in stromal cells that surround the blastocyst during attachment (Song et al., 2000; Stewart et al., 1992). This transient expression of LIF is essential for implantation: females lacking a functional LIF gene are fertile, but their blastocysts fail to implant and do not develop. The blastocysts, however, are viable and, when transferred to wild-type pseudo-pregnant recipients, they can implant and continue embryogenesis (Stewart et al., 1992). LIF binds to the LIF receptor and, as other cytokines, to gp130. Interestingly, deletion of the binding sites of STAT, the gp130 signal transducer and activator of transcription, also results in implantation failure (Ernst et al., 2001). Myc has already been identified as a key target of the LIF/STAT self-renewal pathway: murine ES cells express elevated levels of Myc and following LIF withdrawal, Myc mRNA levels collapse and Myc protein becomes phosphorylated on threonine 58 (T58) and targeted for GSK3β dependent degradation (Cartwright et al., 2005). Along the same line, maintained expression of

a stable Myc mutant (T58A) renders ES cell self-renewal and maintenance of pluripotency independent of LIF (Cartwright et al., 2005). Therefore we are currently investigating the hypothesis that LIF produced in the uterus might induce Myc expression in the embryo rendering it competent for implantation.

Recent publications have heavily questioned the role of Myc in specifying a certain transcriptional signature, and have concluded that Myc simply amplifies a transcriptional program that completely depends on the cell type and its status (Lin et al., 2012; Loven et al., 2012; Nie et al., 2012). An interesting observation is that, independently on the culture conditions, mouse ES cells depend on Myc activity to enter the cell cycle. The close relationship between Myc and mitogenesis has been extensively described both in normal cells and in tumorigenesis (Dang, 2012; Meyer and Penn, 2008) and argues for a specific role for Myc as a general coordinator of cellular proliferation (Littlewood et al., 2012).

The relationship between the cell cycle machinery and the pluripotency network has been the object of several studies but whether the cell cycle regulators can influence pluripotency and differentiation is still not clear. Our data suggest that Myc proteins control the fundamental metabolic pathways of mouse ES cells and are essential for cell cycle progression. The observation that loss of both c- and N-Myc only impairs cell cycle without disrupting the core pluripotency network suggests that maintenance of pluripotency might be at least partially independent on the proliferation status of ES cells.

7. Concluding remarks and outlook

The results reported in this thesis suggest that loss of both c- and N-Myc in naïve mouse ES cells induces a state of metabolic and proliferative quiescence without compromising the expression of the ES cell pluripotency network.

Although Myc dKO ES cells do not activate any apoptotic pathway, they cannot be propagated *in vitro*, most probably as an effect of the metabolic and cell cycle arrest. To elucidate if the quiescent state induced by Myc loss is reversible, we will now induce exogenous Myc expression in dKO ES cells by using a lentiviral inducible system or by direct transfection of *c-myc* mRNA. Gene expression and cell cycle analysis will be used to examine if this exogenous c-Myc activity can rescue the Myc dKO phenotype. This would also demonstrate that the cells were in a reversible state of dormancy rather than in an irreversible cell cycle arrest.

To investigate if Myc genes are the genes regulated *in vivo* during the process of diapause, we will experimentally inhibit embryo implantation by manipulating ovarian estrogen secretion. We will quantify the expression levels of the three Myc family members in normal blastocysts, diapause-embryos and re-activated embryos. Once differences in Myc expression will be confirmed, we will focus on the identification of the factors responsible of Myc regulation. Since previous studies suggest that Myc is a downstream target of the LIF-STAT3 signaling pathway, one of the possible candidates is the cytokine LIF, already shown to be essential for implantation (Wang and Dey, 2006).

We observe that loss of Myc in ES cells results in a significant up-regulation of Dnmt3l. This factor has been recently shown to counteract the activity of the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b to maintain hypomethylation at promoters of bivalent developmental genes (Neri et al., 2013). Therefore we are currently performing DNA methylation analysis by bisulfite sequencing to unravel the global methylation profile of Myc dKO ES cells to address whether the de-

regulation of Dnmt3l is causative for the maintenance of the pluripotency network in Myc mutant ES-cells.

8. Materials and methods

8.1 Mouse lines

Animal experiments were performed according to the GV-Solas (Gesellschaft für Versuchstierkunde). Mice were maintained in the facility of the German Cancer Research Center (DKFZ) in Heidelberg, under specific pathogen-free (SPF) conditions and housed in individually ventilated cages (IVC).

8.1.1 c-myc^{flox} mice

The c-myc^{flox} allele was generated by Andreas Trumpp in the laboratory of Michael Bishop (Trumpp et al., 2001). Two loxP sites flanking the c-myc ORF are located into the 3' untranslated region and into the intron 1 of the c-myc gene. With the use of a Cre-recombinase, the c-myc^{flox} allele can be converted into a knock-out allele (c-myc$^{\Delta}$) (Figure 34).

8.1.2 N-myc^{flox} mice

The N-myc^{flox} allele was generated in the laboratory of Robert Eisenman by inserting *loxP* sites 5' of exon 2 and 3' of exon 3 of the N-myc gene (Knoepfler et al., 2002). Since the translational initiation codon of N-myc is located in the second exon, with the use of a Cre-recombinase the entire coding region of N-myc is floxed, and the N-myc^{flox} allele is converted into a knock-out allele (N-myc$^{\Delta}$) (Figure 34).

8.1.3 REYFP^{flox} mice

The REYFP^{flox} is an allele reporting Cre recombinase activity and is commercially available (Srinivas et al., 2001). In these mice, a loxP-flanked stop codon blocks the expression of the EYFP protein. In the presence of Cre activity, the stop codon is excised, resulting in the constitutive EYFP expression under the Rosa26 promoter (Figure 34).

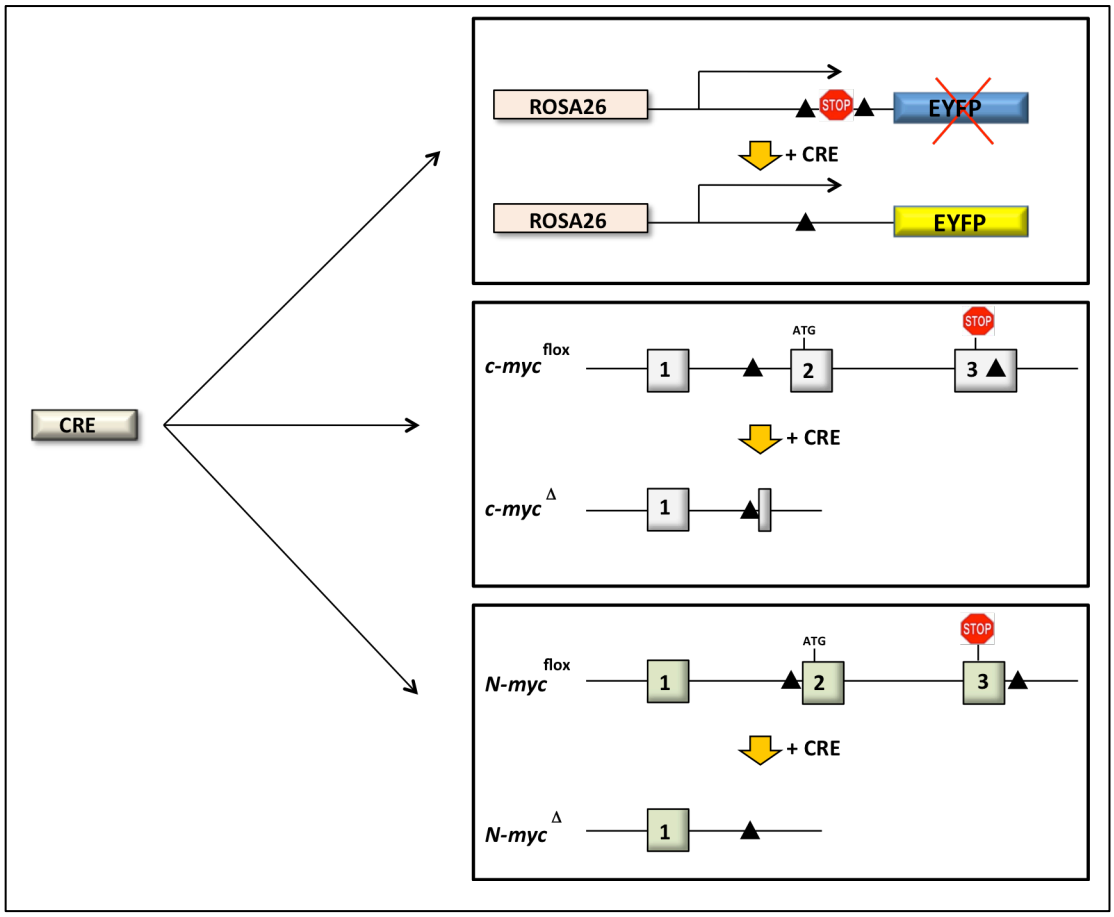

Figure 34: Schematic representation of c-myc^{flox}, N-myc^{flox} and REYFP^{flox} alleles. Upon Cre expression, c-myc and N-myc alleles are recombined and deleted to generate double knockout cells. In a similar fashion, the stop codon located in 5' of the EYFP gene is deleted resulting in constitutive EYFP expression.

8.1.4 c-myc^{GFP} mice

The c-myc^{GFP} allele was generated in the laboratory of Berry Sleckman (Huang et al., 2008). c-myc^{GFP} mice express a GFP-c-Myc fusion protein from the endogenous *c-myc* locus, with GFP appended to the N terminus of c-Myc.

8.1.5 tdTomato mice

The tdTomato mice were generated in the laboratory of Liqun Luo (Muzumdar et al., 2007) and are commercially available (http://jaxmice.jax.org/strain/007576.html). In these mice a membrane-targeted tdTomato (mT) is expressed in all tissues and cell types examined. When bred to Cre recombinase expressing mice, the mT cassette is deleted in the Cre expressing tissue(s), allowing expression of a membrane-targeted EGFP (mG) (Muzumdar et al., 2007).

In this thesis we derived MEF from mice homozygous for the tdTomato allele in order to separate by FACS sorting the feeder layer of MEF from the mouse ES cells.

8.2 Cell culture

8.2.1 Isolation and culture of mouse embryonic fibroblasts (MEF)

MEF Medium

DMEM	500 ml	(Gibco, 11965-092)
ES cells-tested Fetal Calf Serum (10%)	50 ml	(Gibco, 10439)
Non-essential amino acids	5 ml	(Gibco, 11140-035)
Pen/Strep	5 ml	(Gibco, 15140-122)
Glutamine	5 ml	(Gibco, 25030-081)

MEF were obtained from C57BL/6 mice (Harlan). After removing the head and the internal organs, E13.5 or E14.5 embryos were dissociated mechanically and by enzymatic digestion with StemPro-Accutase (Gibco, A11105-01). Cells were grown for 3 to 4 passages on plastic dishes in MEF medium. For use as feeder layers, the cells were mitotically inactivated by exposure to 60 Gy of gamma-irradiation.
Irradiated MEFs were resuspended in medium containing 10% dimethyl-sulphoxide (DMSO) and transferred to cryovials (Nunc, 375418). After over-night storage at -80°C, cells were transferred to liquid nitrogen.

8.2.2 Derivation of mouse ES cell lines from E2.5 morulas

Mouse ES lines were derived in collaboration with the Transgenic Service Core Facility of the German Cancer Research Center (DKFZ). 6-10 weeks old females were injected intraperitoneally with 7 U of pregnant mare serum gonadotrophin (PMSG) followed by 7 U of human chorionic gonadotrophin (hCG), injected 47 hours later. The females were then paired with males and checked for vaginal plug the following morning, considered as 0.5 day postcoitum (dpc). The 2.5 dpc embryos were flushed from the oviducts into a plastic dish with M2 medium (Sigma, M7167). The embryos

were washed 10x in M2 medium. To remove the zona pellucida, the embryos were incubated shortly in Tyrode's solution (Sigma, T1788-100ML) and transferred again to M2 medium. Finally, each embryo was transferred to a well of a 96-well plate previously coated with a layer of mitotically inactivated MEF, in 2i medium plus 15% FCS.

8.2.3 Culture of mouse ES cells

2i + LIF medium

Ndiff N2B27 medium	500 ml	(StemCells, SCS-SF-NB-02)
Pen/Strep	5ml	(Gibco, 15140-122)
b-Mercaptoethanol	500 μl	(Gibco, 31350-010)
LIF (10^3 Units/ml)	500 μl	(Millipore, ESG1106)
PD0325901	1 μM	(StemGent, 04-0006)
CHIR99021	3 μM	(StemGent, 04-0004)

Serum + LIF medium

KO DMEM medium	500 ml	(Gibco, 10829)
Pen/Strep	5ml	(Gibco, 15140-122)
b-Mercaptoethanol	500 μl	(Gibco, 31350-010)
LIF (10^3 Units/ml)	500 μl	(Millipore, ESG1106)
Glutamine	5 ml	(Gibco, 25030)

All mouse ES cell lines were grown on 0.1% gelatin-coated plates (EmbryoMax® 0.1% Gelatin solution, Millipore, ES-006-B) and maintained at 37°C and 5% CO_2 in humidified incubators.

For harvesting, cells were washed in PBS and treated 5 min with StemPro-Accutase (Gibco, A11105-01) detachment solution. Centrifugation steps were carried out at 800g at 4°C for 5 minutes. Cell numbers and viability were determined using a ViCell Counter (Beckman Coulter, Brea, CA).

For long-term storage, ES cells were stored in liquid nitrogen. For freezing, cells were harvested by treatment with StemPro-Accutase. Than 1-3 million cells were

resuspended in 1 ml of medium containing 10% dimethyl-sulphoxide (DMSO) and transferred to cryovials (Nunc, 375418). After over-night storage at -80°C, cells were transferred to liquid nitrogen.

8.3 Fluorescence Activated Cell Sorting (FACS)

FACS analysis was performed on LSRII or LSR Fortessa flow cytometers (Becton Dickinson, San Jose, CA). Data were analysed using the FlowJo software (Tree Star, Ashland, OR).

8.3.1 Cell cycle analysis

For cell cycle analysis, $0.5x10^6$ mES cells were fixed by incubating 20 min at RT in Cytofix/Cytoperm buffer (Beckton Dickinson, 51-2090KZ) and washed in PermWash buffer (Beckton Dickinson, 51-2091KZ). Intracellular staining with Ki76-Alexa647 antibody (BD biosciences, 558615) was performed in PermWash solution over night at 4°C. Samples were than incubated with Hoechst33342 (Sigma, H3570) for 10 minutes and the acquisition was performed immediately after washing.

8.3.2 Expression of pluripotency markers

FACS analysis for the mouse pluripotency factors Nanog, Sox2 and Oct3/4 was performed using the BD™ Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (Becton Dickinson, 560585) according to the manufacturer's instructions. In summary, $1x10^5$ cells were harvested by treatment with StemPro-Accutase and fixed for 20 min at RT in BD Cytofix/Cytoperm buffer. The cells were than permeabilized by washing with PermWash buffer and stained with mNanog-PE, Oct3/4-PerCP-Cy5.5 and Sox2-Alexa Fluor®647 antibodies for 1 hour at 4°C.

8.4 Immunofluorescence

After FACS sorting, $5x10^3$ mES cells were seeded on MEFs in chambered cover glasses (Lab-Tek II, Nunc, 177402) coated with 0.1% gelatin, in 2i + LIF medium. After 72 hours, the cells were fixed by incubating 15 minutes at RT in 4% PFA (Electron

Microscopy Sciences, 15713) and permeabilized in PBS containing 0.5% Triton X-100 for 10 minutes. The cells were than washed three times with PBST (0.1% Tween 20 in PBS) for 5 min at RT. To reduce unspecific binding, the samples were incubated 1 hour in blocking solution (0.5 % BSA, 10% goat serum, 0.1% Triton X-100 in PBS). After blocking, the cells were incubated overnight at 4°C with the anti-Nanog antibody (abcam, ab80892) diluted 1:150 in blocking solution. After washing twice in PBS, the cells were incubated for 4 hours at RT with the secondary antibody goat anti-rabbit-Alexa Fluor 546 (Invitrogen, A-11035) diluted 1:200 in blocking solution. The samples were than washed twice in PBS and stained with DAPI (Sigma, D9542) diluted 1:1000 in PBS. DAPI was incubated for 3 min and samples were washed twice in PBS. The slides were mounted with Faramount (Dako, S3035) and analyzed on a Zeiss Cell Observer fluorescence microscope (Carl Zeiss, Jena, Germany).

8.5 Alkaline Phosphatase (AP) Staining

AP staining was performed using the Stemgent® Alkaline Phosphatase Staining Kit (Stemgent, 00-0055) according to the manufacturer's instructions.

8.6 Molecular biology

8.6.1 DNA extraction

Genomic DNA was isolated from sorted or cultured cells using the DNeasy Blood and Tissue kit (Qiagen, 69506) according to the manufacturer's instructions.

DNA extracts from mouse-tail biopsies were obtained using the Direct PCR Tail Lysis Reagent (Peqlab, 31-102-T).

8.6.2 Genotyping PCR

Genotyping PCRs were performed from cells or tail biopsies following DNA extraction.

Allele	Primer Pair	Primer Sequence	Product Size
c-myc GFP	cmycGFP F1	AAG GGG AGT GGT TCA GGA TT	WT: 290 bp
	cmycGFP F2	AGC AGC TCG AAT TTC TTC CA	GFP: 1000 bp
c-myc flox	5'floxB	ACA ACG TCT TGG AAC GTC AGA GG	WT: 320 bp
	3'floxB	GCA TTT TAA TTC CAG CGC ATC AG	Flox: 450 bp
c-myc delORF	cmycdel1	AAA TAG TGA TCG TAG TAA AAT TTA GCC TG	KO: 190 bp
	cmycdel2	ACC GTT CTC CTT AGC TCT CAC G	
N-myc flox/KO	N-Myc 1	GTC GCG CTA GTA AGA GCT GAG ATC	WT: 217 bp
	N-Myc-2	GGC ACA CAC CTA TAA TCC CAG CTA G	KO: 350 bp
	N-Myc 3	CAC AGC TCT GGA AGG TGG GAG AAA GTT GAGCGT CTCC	Flox: 260 bp
REYFP	Rosa1 (For)	AAA GTC GCT CTG AGT TGT TAT	WT: 600 bp
	Rosa2 (RevEYFP)	GCG AAG AGT TTG TCC TCA ACC	RosaEYFP: 300 bp
	Rosa3 (Rev WT)	GGA GCG GGA GAA ATG GAT ATG	
Tomato-EGFP	Tomato1	CTC TGC TGC CTC CTG GCT TCT	WT: 330 bp
	Tomato2	CGA GGC GGA TCA CAA GCA ATA	Flox: 250 bp
	Tomato3	TCA ATG GGC GGG GGT CGT T	

Table 1: List of PCR primers used for genotyping of mice and cells.

8.6.3 RNA extraction

Cultured cells were harvested by treatment with StemPro-Accutase (Gibco, A11105-01) and cell pellets were lysed in 50 µl of RNA extraction buffer (ARCTURUS® PicoPure® RNA Isolation Kit, Life Technologies, Invitrogen, 12204-01) and stored at -80°C or directly used for RNA extraction using the ARCTURUS® PicoPure® RNA Isolation Kit, according to the manufacturer's protocol and including an on-column DNase digestion (Qiagen, 79254) to remove genomic DNA.

8.6.4 Reverse transcription

Reverse transcription was performed using the SuperScript VILO cDNA synthesis kit (Invitrogen, 11754-250) according to the manufacturer's instructions. 0.1-2 µg of RNA were used as template and cDNA was diluted in DNase free water (Gibco, 10977) and used for qRT-PCR.

8.6.5 qRT-PCR

Real-time quantitative PCRs were performed using the ABI Power SYBR Green Master Mix (Applied Biosystems, 4309155) in reaction volumes of 10 µl. PCR reactions were run on a Viia7 machine (Applied Biosystems) with the following program: 95°C, 10 min; 50 cycles (95°C, 15 sec; 60°C, 45 sec; acquisition); 95°C, 10 sec; 65°C, 1 min; 0.11°C/s up to 95°C continuous acquisition; 40°C, 5 min. Results were analyzed using the Viia^{TM}7 –software 1.1.

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
c-myc	CAC CAG CAG CGA CTC TGA	GGG GTT TGC CTC TTC TCC
N-myc	CTC CGG AGA GGA TAC CTT GA	TCT CTA CGG TGA CCA CAT CG
L-myc	CCA GCG ATT CTG AAG GTG A	TCC AGA GAT CGC CTC TTC TC
Nanog	TTC TTG CTT ACA AGG GTC TGC	AGA GGA AGG GCG AGG AGA
Oct4	GTT GGA GAA GGT GGA ACC AA	CTC CTT CTG CAG GGC TTT C
Klf4	CGG GAA GGG AGA AGA CAC T	GAG TTC CTC ACG CCA ACG
Rex1	TCT TCT CTC AAT AGA GTG AGT GTG C	GCT TTC TTC TGT GTG CAG GA
p57	TCT CGG GGA TTC CAG GAC	ACG TTT GGA GAG GGA CAC C
P21	TCC ACA GCG ATA TCC AGA CA	GGA CAT CAC CAG GAT TGG AC
P27	GAG CAG TGT CCA GGG ATG AG	TCT GTT CTG TTG GCC CTT TT
P19	CGG TAT CCA CTA TGC TTC TGG AA	CCG CTG CGC CAC TCA
Gapdh	CCC ATT CTC GGC CTT GAC TGT	GTG GAG ATT GTT GCC ATC AAC GA
SDHA	AAG TTG AGA TTT GCC GAT GG	TGG TTC TGC ATC GAC TTC TG
OAZ1	TTT CAG CTA GCA TCC TGT ACT CC	GAC CCT GGT CTT GTC GTT AGA
DNMT1	TTT ACG TGT CGT TTT TCG TCT C	CAG AGA CTC CCG AGG ACA GA
DNMT3a	AAA CGG AAA CGG GAT GAG T	ACT GCA ATT ACC TTG GCT TTC T
DNMT3a2	ACA CAG GGC CCG TTA CTT CT	TCA CAG TGG ATG CCA AAG
DNMT3b	GAA CAT GCG CCT GCA AGA	GCA CAG ACT TCG GAG GCA AT
DNMT3l	CGG CAC CAG CTG AAG GCC TTC CAT G	AGG CAG CGC ATA CTG CAG GAT CCG G

Table 2: List of primers used for qRT-PCR experiments.

All primers were designed using the Roche Universal Probe Library Assay Design Center. c-myc, N-myc and L-myc primers were designed by Dr. Armin Ehninger and

were validated with defined molecular amounts of template DNA to enable comparative quantification. GAPDH, OAZ1 and SDHA were used for normalization. The primers used in this thesis are listed in Table 2.

8.6.6 Cloning of pBS598 ef1α mCherry-Cre plasmid

The pBS598-ef1α-mCherry-Cre plasmid (mCherry Cre) carrying the mCherry fluorescent protein in frame with the Cre recombinase has been generated starting from the pBS598-EF1a-EGFP-Cre plasmid (Addgene, 11923). This plasmid has been cut with the NcoI restriction enzyme to eliminate the EGFP coding sequence and dephosphorylated to avoid recircularization. The mCherry ORF has been obtained via PCR using the pmCherry-NLS plasmid (Addgene, 39319) as template and the following custom primers carrying the BspHI restriction size (shown in bold):

mCHERRYup: AAA**TCATGA**TGGTGAGCAAGGGCGA

mCHERRYdw: AAA**TCATGA**CCTTGTACAGCTCGTCCATGC

The PCR product has been purified with phenol/chloroform, cut with BspHI, gel purified and ligated to the NcoI-treated pBS598-EF1a-EGFP-Cre plasmid. The use of the BspHI enzyme, that carries NcoI compatible ends, has been necessary since the mCHERRY ORF includes an NcoI restriction site making impossible a direct sub-cloning. The map of the pBS598-ef1α-mCherry-Cre is reported in Figure 35.

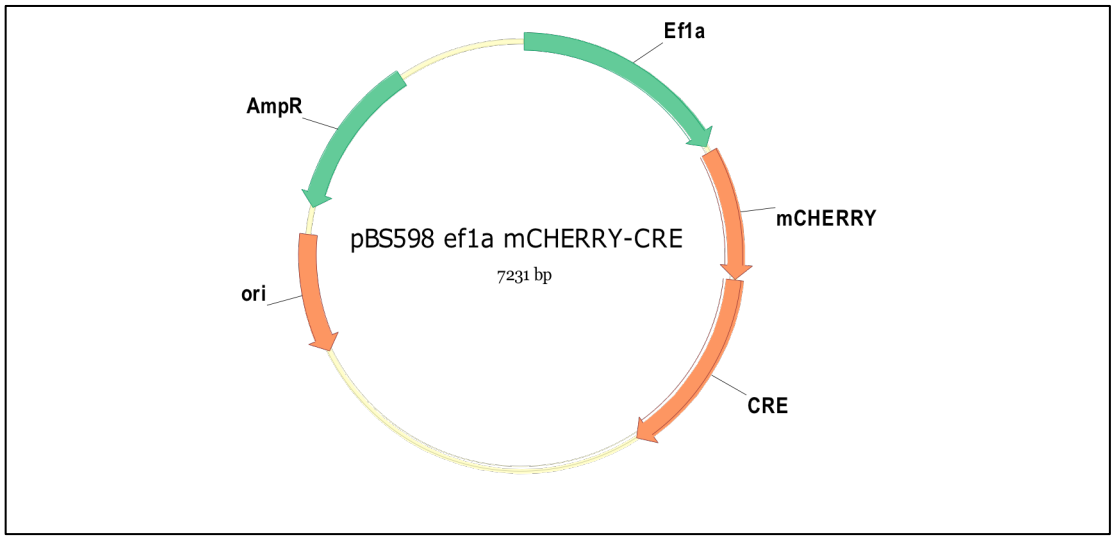

Figure 35: Map of the pBS598-ef1α-mCherry-Cre plasmid.

8.7 Teratoma Assay

8.7.1. Subcutaneus injection of mES cells

For teratoma assay, ES cells were harvested and resuspended in PBS at a density of $6x10^6$ cells/ml. $3x10^6$ ES cells were injected subcutaneously into the flank of 6-weeks old female NSG immunodeficient mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory). 4-6 weeks after injection, when the tumors reached approximately 1.5 cm in diameter, the mice were sacrificed and teratomas were isolated and further processed.

8.7.2. Hematoxylin-Eosin (H&E) staining

After surgical removal, the tumors were fixed in 10% buffered formalin solution (Sigma, HT-1-2) for 2 days, at 4°C on a rotor. The Tissue-Tek®VIP®6 was used to dehydrate the samples by sequential incubation in 70% - 90% and 100% ethanol, followed by treatment with xylol and paraffin. Paraffin-embedded tissues were then cut in 4 µm sections (Microm HM355S microtome, Thermo Scientific Inc., Bremen, Germany) and then deparaffinized and rehydrated by sequencial incubation in xylol (twice, for 3 minutes) and in alcohol (100% - 95% - 80% - 70% and 50% ethanol, for 3 minutes each). The samples were stained with hematoxylin for 6 minutes, washed in water, briefly immersed in acidic ethanol (200 ml 70% ethanol + 0.5 ml pure HCl) and then counter-stained with eosin. After short immersion in 95% and 100% ethanol, the samples were incubated in xylol for 15 minutes and finally embedded. After air-drying, embedded slides were stored at room temperature and imaged using the Leica DM LB2 microscope (Leica Microsystems, Germany).

8.8 RNA-sequencing

8.8.1. Cell culture, transfection and FACS sorting

c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; ROSA-EYFP$^{flox/flox}$ ES cells were maintained in 2i + LIF medium for more than 10 passages. For the deletion of the Myc alleles, $1x10^5$ cells were plated in each well of a 6-well plate, pre-coated with 0.1% gelatin. After 10 hours the cells were transfected with the mCherry-Cre plasmid by using the Xfect™

Stem kit (Clontech, 631321) according to the manufacturer's instructions. 24 hours after transfection, ES cells were harvested and resuspended in ice-cold PBS.
Cell sorting was performed on a FACS Aria II or FACS Aria III (Becton Dickinson, San Jose, CA) at the DKFZ Flow Cytometry Service Unit, Heidelberg, Germany. The following sort parameters were used: 85 µm nozzle; maximum 10,000 evt/s.; 45 psi. Sorted cells were collected into ice-cold PBS, span down and seeded into 24-well plates at a density of $5x10^4$ cells/well, or resuspended in 100 µl of RNA extraction buffer (ARCTURUS® PicoPure® RNA Isolation Kit, Life Technologies, Invitrogen, 12204-01) and stored at -80°C until further usage. Seeded cells were harvested 72 hours after sort for RNA isolation.

c-Myc GFP ES cells were isolated from eGFP-c-Myc knocking mice . Clones C6, D7 and E4 were used as three biological replicates and were maintained for more than 10 passages in 2i + LIF medium. For the RNA-sequencing experiment, ES cells were plated at a density of $0.5x10^6$ cells/dish in 10 cm dishes pre-coated with tdTomato MEFs, in 2i + LIF or serum + LIF medium. The medium was freshly replaced every day. 72 hours after seeding, the cells were harvested and resuspended in PBS at a concentration of $10x10^6$ cells/ml. For dead cell exclusion, PI (Sigma, P4864) was added at a 1:100 dilution.
Cell sorting was performed on a FACS Aria II or FACS Aria III (Becton Dickinson, San Jose, CA) at the DKFZ Flow Cytometry Service Unit, Heidelberg, Germany. The following sort parameters were used: 85 µm nozzle; maximum 10,000 evt/s.; 45 psi. Sorted cells were collected into ice-cold PBS, span down and resuspended in 100 µl of RNA extraction buffer (ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies, Invitrogen, 12204-01) and stored at -80°C until further usage.

8.8.2. Total RNA isolation and RNA-sequencing

Total RNA isolation was performed using ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies, Invitrogen, 12204-01) according to the manufacturer's instructions. To remove genomic DNA, we performed a DNAse treatment using the RNAse-free DNAse Set (Qiagen, 79254). Total RNA was used for quality controls and

for normalization of starting material. For the Myc dKO RNA-seq, cDNA-libraries were generated with 10 ng of total RNA using the SMARTer™ Ultra Low RNA Kit for Illumina Sequencing (Clontech) according to the manufacturer's indications. Of note, 12 cycles were used for the amplification of cDNA. Paired-end adaptors were applied to each population. 100 bp reads sequencing was performed with the HiSeq2000 device (Illumina) and three samples per lane.

8.8.3. Processing of RNA-seq raw data

Bioinformatic analysis of the RNA-seq data was performed by Alejandro Reyes (EMBL, Heidelberg). Read fragments were aligned against the mouse reference genome GRCm38 from ENSEMBL release 69, using GSNAP (version 2012-07-20). Splitted alignments were allowed for exon-exon junctions. Only uniquely aligned fragments were considered for further analysis. For each gene, the number of read fragments was calculated using htseq-count. The likelihood ratio test from DESeq2 was used in order to test for differential expression in any of the conditions. The variance stabilizing transformation from DESeq2 was used to make the data homoscedastic through the dynamic range. A background matched for expression strength with the differentially expressed genes was generated to avoid any bias associated to expression strength in further testing. The variance stabilized data were considered in order to group the differentially expressed genes by their relative expression level compared to the mean expression across all the conditions. Gene Onthology enrichment tests were performed compared to the background genes using a Fisher's exact test. Multiple-testing correction was done with the Bonferroni-Hochberg method. Principal component analysis and hierarchical clustering were done with R.

9. Abbreviations

2i	two inhibitors
3i	three inhibitors
AP	alkaline phosphatase
BMP	bone morphogenic protein
BSA	bovine serum albumin
cDNA	complementary DNA
CDK	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CHIR	CHIRON99021
dKO	double knock-out
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
Dpc	days post coitum
EC	embryonal carcinoma
ER	estrogen receptor
ERK	extracellular receptor kinase
ES	embryonic stem
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	FGF receptor
FITC	fluorescin isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorecent protein
GSK3b	glycogen synthase kinase 3b
H&E	hematoxylin and eosin
hCG	human chorionic gonadotrophin

HSCs	hematopoietic stem cells
ICM	inner cell mass
IL-6	interleukin 6
iPS	induced pluripotent stem
JAK	janus activated kinase
Klf	Kruppel-like family
LIF	leukaemia inhibitory factor
MAPK	mitogen activated protein kinase
MEFs	mouse embryonic fibroblasts
mRNA	messenger RNA
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD	PD0325901
PcG	polycomb
PI	propidium Iodide
PI3K	phosphatidylinositol 3-kinase
Pol	polymerase
PMSG	gonadotrophin
qRT- PCR	quantitative real-time PCR
RNA	ribonucleic acid
RNA-seq	RNA-sequencing
RNAi	RNA-interference
siRNA	short interfering RNA
SSNT	somatic cell nuclear transfer
STAT	signal transducer and activator of transcription
trxG	trithorax
wt	wild type

10.Contributions

The c-myc$^{flox/flox}$; N-myc$^{flox/flox}$; REYFP$^{flox/flox}$ and c-myc$^{GFP/GFP}$ embryonic stem (ES) cell lines were generated by Frank van der Hoeven (DKFZ, Heidelberg) and myself, in the transgenic service unit of the German Cancer Research Center.

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Dr. Nina Cabezas (DKFZ, Heidelberg) provided scientific support for the RNA-sequencing. Alejandro Reyes (EMBL, Heidelberg) performed the bioinformatic analysis of the data.

Dr. Sandro Altamura (MMPU, Heidelberg) cloned the pBS598 ef1α mCherry-Cre plasmid, provided the E14 ES cells and critically reviewed this thesis.

Cell sorts were performed in collaboration with Gelo de la Cruz and Ann Atzberger at the DKFZ flow cytometry core facility.

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