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myc maintains embryonic stem cell pluripotency and self-renewal

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Abstract

While endogenous *Myc* (c-*myc*) and *Mycn* (N-*myc*) have been reported to be separately dispensable for murine embryonic stem cell (mESC) function, *myc* greatly enhances induced pluripotent stem (iPS) cell formation and overexpressed c-*myc* confers LIF-independence upon mESC. To address the role of *myc* genes in ESC and in pluripotency generally, we conditionally knocked out both c- and N-*myc* using *myc* doubly homozygously floxed mESC lines (cDKO). Both lines of *myc* cDKO mESC exhibited severely disrupted self-renewal, pluripotency, and survival along with enhanced differentiation. Chimeric embryos injected with DKO mESC most often completely failed to develop or in rare cases survived but with severe defects. The essential nature of *myc* for self-renewal and pluripotency is at least in part mediated through orchestrating pluripotency-related cell cycle and metabolic programs. This study demonstrates that endogenous *myc* genes are essential for mESC pluripotency and self-renewal as well as providing the first evidence that myc genes are required for early embryogenesis, suggesting potential mechanisms of *myc* contribution to iPS cell formation.

Keywords

Embryonic stem cells; Myc; iPS cells; Pluripotency; Self-renewal

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1. Introduction

myc proto-oncogenes encode transcription factors belonging to the superfamily of basic helix-loop-helix (bHLHZ) proteins. The three main *myc* family members are involved in fundamental normal cellular processes including proliferation, differentiation, and apoptosis (Meyer and Penn, 2008). Constitutive targeted disruption of c-*myc* causes embryonic lethality around E10.5 with embryos exhibiting hematopoietic and vascular defects (Davis et al., 1993). c-*myc* has also been shown to play a pivotal role in B and T cell development (Douglas et al., 2001; Trumpp et al., 2001). Embryos, constitutively lacking N-*myc*, die before E11.5 and display a host of defects including disrupted neuroectodermal, heart, and lung development (Stanton et al., 1992; Charron et al., 1993; Nagy et al., 1998; Moens et al., 1992). Conditional knockout of N-*myc* in neural stem and progenitor cells (NSC) leads to a profound disruption of brain growth attributable in part to altered NSC cell cycling (Knoepfler et al., 2002). Although widely expressed in the murine embryo, L-*myc* appears to be dispensable for embryonic development (Hatton et al., 1996). Together these studies indicate that both c- and N-*myc* are key regulators of embryogenesis from midgestation onward, but leave open the question of potential roles for *myc* genes in early embryonic development. Surprisingly, separate disruption of c- and N-*myc* had no reported effect on mESC biology (Davis et al., 1993; Sawai et al., 1991). The lack of phenotype of the single KOs may be explained by the fact that c- and N-*myc* appear to be highly redundant (Malynn et al., 2000).

Recently, interest in *myc* function in stem cells was reignited by studies linking both c- and N-*myc* to the generation of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Okita et al., 2007; Sridharan et al., 2009), to the regulation of LIF signaling in mESC (Cartwright et al., 2005; Bechard and Dalton, 2009), and to tumor stem cells as well (Wang et al., 2008). iPS cells have subsequently been produced by many other groups (Yamanaka, 2009) and in the course of these studies, although iPS cell production without ectopic c-*myc* has been reported (Nakagawa et al., 2008; Yu et al., 2007), efficiency was dramatically reduced highlighting an important role for *myc* in establishing pluripotency. Subsequently, genomics studies have suggested that the reprogramming is inefficient in the absence of ectopic c-*myc* expression because c-Myc acts to repress fibroblast-specific gene expression, an event that is predicted to be important early in the reprogramming process (Sridharan et al., 2009). Collectively, these findings indicate there are critical, if yet to be fully defined roles for *myc* genes in self-renewal and pluripotency.

We hypothesized that the single KO studies of c- and N-*myc* in mESC failed to reveal their functions in ESC due to the remaining presence of the other main *myc* gene. To test this hypothesis and analyze *myc* function in ESC as well as in early embryogenesis, we have simultaneously disrupted both c- and N-*myc* using two novel *myc* doubly homozygously floxed (c-$myc^{flox/flox}$; N-$myc^{flox/flox}$) mESC lines, into which Cre recombinase was introduced. After knockout of c- and N-*myc*, both cDKO mESC lines exhibited profound disruption of pluripotency and selfrenewal. Our studies demonstrate that loss of c- and N-*myc* not only triggers growth inhibition due to cell cycle arrest and an increase in apoptosis, but it also strongly induces differentiation into ectoderm, mesoderm, and endoderm derivatives. Gene expression signatures indicative of more advanced differentiation into a number of lineages including neuronal, sensory organ, and hematopoietic were also evident. c- and N-*myc* are also essential for early embryogenesis as chimeric embryos formed with microinjected *myc* DKO mESC most often failed to develop and when they did form, they had very severe defects. These results indicate that *myc* genes are crucial for maintenance of pluripotency and self-renewal of mESC both in vitro and in vivo, with important implications for iPS cells and tumor stem cells.

2. Materials and methods

2.1. Myc doubly floxed mES cell line generation

Twenty-one blastocysts and eighty-two morula were harvested from either seven 3-week-old or four 6-week-old *myc* doubly floxed superovulated female mice that had been crossed with *myc* doubly floxed males. The blastocysts were immediately seeded onto mouse embryonic fibroblasts (MEFs) in KOSR medium, and the morula were cultured in KSOM medium overnight to attain the blastocyst stage and then seeded onto MEFs. Forty-five of the sixty-eight embryos developed ICM outgrowths that were disaggregated into ESC media and passaged to derive fifteen lines, five of which yielded metaphase spreads that had 80% or greater normal karyotype. Out of five newly generated *myc* doubly floxed lines, two lines (cDKO2 and cDKO3) were chosen for further analysis.

2.2. Cell culture and retroviral transduction

Cells were grown in ES complete medium consisting of high glucose DMEM supplemented with 15% FBS (HyClone), 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 1 × Glutamax, 1 × NEAA (Gibco), with 1000 U/ml LIF (ESGRO, Chemicon) on feeder layers of irradiated MEF cells (Millipore). Retroviral work was conducted as described (Takahashi et al., 2007). cDKO-2 and cDKO-3 mESC lines displayed normal morphology, expression of SSEA-1, Oct3/4, Nanog, Alkaline phosphatase (AP) activity, and normal karyotype (Supplementary Figs. S1 and S2).

2.3. Genotyping and gene copy number analysis by qPCR

GFP positive mESC were sorted using the inFlux Cell Sorter (BD Biosciences). Genomic DNA was isolated from the sorted ESC using PureLink Genomic DNA purification mini kit (Invitrogen). Genotyping for c- and N-*myc* was conducted using multiplex PCR with single reactions that can strongly detect WT, floxed, and null alleles. Primers for c- and N-*myc* are listed in Supplementary Table S2.

Real-time qPCR for detection of gene copy number changes was performed using SYBR Green I detection as described (Hoebeeck et al., 2007). Primers are listed in Supplementary Table S2.

2.4. Immunofluorescence

Cells were fixed with 4% paraformaldehyde in phosphatebuffered saline (PBS) for 20 min. Cells then were stained using mouse anti-SSEA-1 (Chemicon), rabbit anti-GATA6 (Abcam), rabbit anti-alpha smooth muscle Actin (Abcam), mouse anti-α-fetoprotein (R and D systems), mouse anti-TUJ1 (Covance), rabbit anti-Bmp4 (Abcam), rabbit anti-neurogenin3 (Abcam), and Alex-aFluor 546-conjugated secondary antibodies (Invitrogen) following the manufacturer's protocols. AP staining was performed using the alkaline phosphatase substrate kit III (Vector Laboratories). Cells were visualized by a Nikon ECLIPSE 80i fluorescent microscope.

2.5. Flow cytometry

For the analysis of apoptotic cells, mESC were stained for Annexin V-APC (BD Pharmingen) following the manufacturer's protocol. For the analysis of SSEA-1 expression, cells were incubated with PE-conjugated anti-SSEA-1 antibody (R and D systems) or with PE-conjugated mouse isotype control IgM antibodies for 30 min at 4 °C. FACS analysis was performed using a CyAn ADP flow cytometer (Dakocytomation) and data was analyzed using Summit V 4.3 software. For the cell cycle analysis, mESC were pulsed with 10 μM BrdU (BD Pharmingen) for 30 min. BrdU incorporation assays were performed using the

BrdU APC Flow Cytometry kit (BD Pharmingen), according to the manufacturer's instructions. FACS analysis was performed using a LSRII flow cytometer (BD Biosciences).

2.6. Chimeric embryo assays

GFP and GFP-Cre transduced mESC (sorted using the inFlux Cell Sorter, BD Biosciences) were microinjected into early stage embryos (8 cell, morula, or blastocysts), with the stage matched between GFP and GFP-Cre transduced mESC. Total numbers of injected embryos from 2 independent experiments were as follows: WT GFP: 29, WT GFP-CRE: 38, L2 GFP: 30, L2 GFP-CRE: 41, L3 GFP: 45, L3 GFP-CRE: 53. Microinjected embryos were transferred to pseudopregnant females and allowed to develop for 8–9 days before harvesting and microscopic analysis.

2.7. RT-PCR, qPCR, and gene expression analysis

GFP positive mESC were sorted using the inFlux Cell Sorter (BD Biosciences). Total RNA was isolated from the sorted ESC using the RNeasy Plus mini kit (Qiagen, Catalog #74134) as described by the manufacturer. RNA was reverse-transcribed by using Superscript III First strand Synthesis Supermix (Invitrogen). Primer sequences for PCR are listed in Supplementary Table 2.

qPCR assays were performed in triplicate using Invitrogen Express qPCR Supermix with Applied Biosystems Taqman assays on a Roche LightCycler 480. Expression was normalized using Eif4g2 (Nat1). Sample data was analyzed using the comparative Ct method and standard deviation calculated based on Applied Biosystems methods (Bulletin 04371095).

For gene expression analysis, isolated RNA (40 ng/μl) was submitted to the UC Davis Expression Analysis Core for gene expression analysis using the Illumina Sentrix Expression Beadchip (Mouse-6 v.3). Obtained results were analyzed with DAVID 2008 Functional Annotation Bioinformatics Microarray software.

3. Results

3.1. Loss of c- and N-myc disrupts mESC pluripotency, triggering lineage commitment

To examine the function of *myc* genes in mESC self-renewal and pluripotency as well as in early embryogenesis, we created two novel *myc* doubly homozygously floxed (c-*myc*$^{flox/flox}$; N-*myc*$^{flox/flox}$; Fig. 1A and Supplementary Fig. S1A) mESC lines, cDKO-2 and cDKO-3. Both cDKO lines were characterized as having a normal karyotype and pluripotency marker expression prior to use (Supplementary Figs. S1 and S2). To produce a double *myc* knockout (DKO), the cDKO lines were transduced with an MSCV retrovirus containing a GFP-ires-Cre (GFP-Cre) recombinase bicistron. We have used this virus in the past for efficient *myc* KO in vitro in NSC (Knoepfler et al., 2002). We employed quantitative genomic qPCR to measure c- and N-*myc* gene copy numbers and found that they were reduced approximately 4-fold in cDKO lines transduced with GFP-Cre, but not in WT lines transduced with GFP-Cre (Fig. 1B). After 5 days of Cre expression, cDKO mESC exhibited a significant inhibition of colony growth (Fig. 1C, Supplementary Fig. S3A and B), while Cre had no discernable effect on WT mESC. Thus, infecting cDKO-2 and cDKO-3 mESC lines with GFP-Cre virus is a very efficient method of conditional *myc* gene disruption.

To test whether simultaneous depletion of c- and N-*myc* disrupts colony growth by impairing pluripotency, we analyzed the expression of differentiation markers in the two cDKO mESC lines by RT-PCR, qRT-PCR, and immunofluorescence analysis. cDKO-2 and cDKO-3 cells transduced with Cre exhibited 3–5 fold decreases in c- and N-*myc* levels (Fig.

1D). Because Myc RNA and proteins are known to have extremely short half-lives with a typical half-life of approx. 30 min, Myc protein levels in these cells should exhibit similar decreases (Hann and Eisenman, 1984). The *myc*-deficient cells also showed significant induction of a variety of lineage markers such as *Bmp4*, *gata6*, *Fgf5*, and *Ngn3* relative to GFP control transduced mESC (Fig. 1D, Supplementary Figs. S4 A and B).

3.2. Loss of myc drives differentiation-associated gene expression in mESC strongly including expression of hematopoietic and neuronal/sensory organ lineage genes

Microarray gene expression experiments were performed at days 5 and 7 of Cre expression in cDKO lines to capture the dynamic changes in the mESC transcriptome associated with the loss of *myc*. We found that 437 genes after 5 days of CRE expression and that 688 genes after 7 days of CRE expression were up-regulated at least 1.5-fold in both cDKO mESC lines, but not in the WT cell line. Gene expression profiles of cDKO cell lines revealed a significant increase in expression of differentiation markers such as *Ngn3*, *Fgf5*, *Bmp4*, and *gata*6, which confirmed our initial RT-PCR and qRT-PCR data (Supplementary Table S1, Supplementary Fig. S4A, and Fig. 1D). Importantly, ontological functional annotation analysis (http://david.abcc.ncifcrf.gov/) of the genes up-regulated in both cDKO-2 and cDKO-3 lines with 5 days of Cre expression revealed upregulation of 15 genes involved in hematopoietic differentiation ($p=2.2\times10^{-4}$) including *Vav1*, *Egr1*, and *Epas-1* (Supplementary Table S1). The loss of *myc* also triggered upregulation of 19 transcriptional activators ($p=1.2\times10^{-4}$) including *Stat1*, *Egr1*, and *Elk3*, which are known to drive hematopoietic differentiation (Supplementary Table S1). These data suggest that loss of *myc* drives mESC to differentiate toward hematopoietic lineages, further supported by analysis of the genes up-regulated in the cDKO cell lines after 7 days of Cre expression. Twelve up-regulated genes were involved in lymphocyte differentiation ($p=1.9\times10^{-3}$) and 26 genes were linked to the regulation of immune system processes ($p=1.4\times10^{-2}$), such as *cd4*, *cd80*, and *Fcgr2b* (Supplementary Table S1). These data also indicate that some mESC show gene expression signatures of progression toward advanced stages of hematopoietic, and more specifically, lymphocytic differentiation. However, due to continued expression of only modestly reduced levels of pluripotency markers such as Oct4, normal, and terminal hematopoietic differentiation is unlikely to fully occur in the cDKO cultures (Supplementary Fig. S4D), fitting with the general absence of other mature lineage markers such as SMA and low levels of TUJ1.

In addition to lymphocyte activation and differentiation, both lines of *myc*-deficient cells exhibited pronounced activation of markers specific to sensory organ development ($p=6.9\times10^{-4}$), including *Dll1*, *Bmp4*, *Gbx2*, and *Fgfr1* genes, as well as genes specific to the lens (*Crygc* and *Crygd*) and the inner ear (*Cdh23*) (Supplementary Table S1). The array data were validated by conventional and qRT-PCR (Fig. 1D and E). Importantly, *myc* DKO also led to a pronounced upregulation of Wnt pathway genes ($p=2\times10^{-3}$), which can partially substitute for *myc* activity in iPS cell formation (He et al., 1998; Marson et al., 2008).

3.3. The pattern of genes downregulated with loss of myc suggests important roles in cellular metabolism, chromatin, and Lif signaling

Fewer genes were downregulated in *myc*-deficient cells than up-regulated: 131 genes were downregulated after 5 days of Cre expression and 255 genes were downregulated after 7 days of Cre expression. Only four genes were downregulated at both 5 and 7 days post-transduction in both cDKO lines: *Klhl34*, *Espn*, *Gnmt*, and, importantly, *Lif*, encoding the key mESC growth factor. Amongst the genes whose expression was downregulated at either days 5 or 7, but not necessarily both, there was down-regulation of genes involved in cellular chemical homeostasis ($p=5.6\times10^{-3}$) and genes regulating organelle biogenesis ($p=4.4\times10^{-2}$), indicating that *myc* DKO leads to down-regulation of general metabolic

activity in mESC (Supplementary Table S1). The loss of *myc* also leads to down-regulation of several helicases including *Chd1*, which regulates open chromatin and pluripotency of ESC, suggesting a role for *myc* in remodeling of ESC chromatin architecture (Supplementary Table S1) (Gaspar-Maia et al., 2009).

3.4. myc depletion disrupts mESC self-renewal, leading predominantly to the earliest stages of differentiation with rarer fully differentiated cells

Growth inhibition of *myc* cDKO mESC was accompanied by significant reduction in the expression of the early embryonic stem cell marker, SSEA-1, indicative of the loss of self-renewal (Fig. 2A and B). Similarly, alkaline phosphatase (AP) assays demonstrated a strong decrease in self-renewal in both *myc*-deficient mESC lines (Fig. 2C and D). Furthermore, approximately 70% of myc-deficient mESC colonies expressed GATA-6 or BMP4 differentiation markers suggesting that the majority of the colonies have entered an early stage of cell determination or differentiation (Figs. 2E and 3). To determine the percentage of differentiating cells upon *myc* knockdown we analyzed the expression of early (Gata6, Bmp4, and Ngn3) and late (β-TUJ, SMA, and AFP) lineage markers by immunostaining (Figs. 3 and 4). Approximately 70% of *myc*-deficient cells were expressing Gata6, a transcription factor expressed in primitive endoderm and cardiac mesoderm, and Bmp4, which controls mesoderm differentiation in early differentiating ES cells as well as promotes ectoderm differentiation at the expense of neural ectoderm in more differentiated ES cells (Figs. 2E and 3) (Cai et al., 2008; Peterkin et al., 2003; O'Shea 2004). Approximately 20% of *myc*-deficient cells expressed Ngn3, which is involved in neuroectoderm differentiation, and is expressed in endocrine progenitor cells (Fig. 3) (Gradwohl et al., 2000). In contrast, there was no detectable expression of the late differentiation markers SMA (smooth muscle cells) and AFP (visceral endoderm), but interestingly 5% of *myc*-deficient cells expressed TUJ1, a mature neuronal marker (Fig. 4). Although *myc* disruption triggered expression of differentiation markers, the mRNA levels of pluripotency regulators including *Nanog*, *Oct4*, and *Rex-1*, were not detectably changed (Supplementary Fig. S4C). Protein levels of Sox2 and Oct4, however, did show a modest decrease in cDKO lines after 5 days of CRE expression measured by FACS, indicative of posttranscriptional modulation of pluripotency factor expression (Supplementary Fig. S4D). Nanog levels were unchanged demonstrating that the decreases in Sox2 and Oct4, while moderate, are specific (data not shown). Passaging of *myc* cDKO mESC infected with GFP-Cre virus demonstrated a significant reduction of GFP+subpopulations, validating the loss of self-renewal (Supplementary Fig. S3A and B), while cDKO mESC transduced with GFP control virus as well as WT cells infected with either GFP or GFP-Cre virus exhibited robust, unaltered self-renewal. Our studies thus demonstrate that c- and N-*myc* are essential for maintaining mESC self-renewal.

3.5. myc regulates mESC self-renewal through promoting S phase progression

Myc genes have well-characterized roles in cell cycle control and apoptosis in a variety of cell types including somatic stem cells (Wang et al., 2008; de Alboran et al., 2001). Knockdown of c-*myc* in glioma cancer stem cells also resulted in cell cycle arrest in the G_0/G_1 phase and increased apoptosis (Wang et al., 2008). While the loss of *myc* strongly impairs cell cycle progression and self-renewal of somatic stem cells (Knoepfler et al., 2002; Wilson et al., 2004), roles for endogenous *myc* genes in mESC cell cycle have not previously been directly addressed. Here we found that c- and N-*myc* play an important role in the maintenance of mESC normal cell cycling. *myc* cDKO mESC exhibited increased expression of 14 genes involved in the regulation of progression through the cell cycle as early as 5 days of Cre expression ($p=1.9\times10^{-2}$; Supplementary Table S1) including *cdkn1c*, encoding a cyclin-dependent kinase inhibitor also called p57, which is essential for TGFβ-induced cell cycle arrest (Scandura et al., 2004), and *gadd45g*, the G_2/M growth arrest gene, a known Myc target (Marhin et al., 1997). The increase in *p57* (*cdkn1c*) and *gadd45g*

expression in *myc* cDKO cell lines was confirmed by real-time qRT-PCR (Supplementary Fig. S5).

We analyzed cell cycle profiles of the *myc* cDKO cell lines by measuring bromodeoxyuridine (BrdU) incorporation coupled to the staining of DNA content with 7-amino-actinomycin D (7-AAD). Flow cytometric analysis revealed 27–31% decreases in the fraction of cells in S phase with concomitant increase of the G_0/G_1 and the G_2/M cell populations (Fig. 5A) in the *myc* cDKO mESC lines. These results indicate that *myc* facilitates progression through S phase and the G_2-M checkpoint in mESC.

3.6. Loss of myc results in a modest increase in apoptosis in mESC

We observed an increase in the expression of genes involved in induction of apoptosis ($p=3.8\times10^{-2}$) in the gene expression patterns associated with the *myc* cDKO by ontological analysis (Supplementary Table S1). To examine potential links between *myc* and mESC survival, we measured apoptotic cell populations following the depletion of *myc* using Annexin V staining. These experiments indicated that loss of *myc* triggered a 3-fold increase in the levels of apoptosis after 7 days of Cre expression (Fig. 5B), while similar results were obtained at day 5 (not shown). Taken together these results demonstrate a role for *myc* as a survival factor for mESC, but the absolute levels of apoptosis even in DKO mESC remain relatively low, suggesting the failure of *myc* DKO mESC to grow is predominantly due to loss of self-renewal and pluripotency. Interestingly, c- and N-*myc* DKO in hematopoietic stem cells also induces apoptosis, however in a much more pronounced manner than we observed here in mESC (Laurenti et al., 2008).

3.7. c- and N-myc are essential for early embryogenesis

To investigate the role of c- and N-*myc* in early embryogenesis, chimeric embryo assays were conducted. GFP and GFP-Cre transduced WT and *myc* cDKO mESC were, in parallel, micro-injected into blastocysts and earlier embryos, followed by transplantation into pseudopregnant hosts (Supplementary Fig S6A). Embryos were harvested for analysis 8–9 days later around midgestation. Microinjection of GFP-Cre transduced WT mESC actually produced more surviving normal midgestational embryos than GFP control transduced WT mESC, and these embryos did not show any developmental abnormalities or reduction in the degree of ESC contribution to embryonic tissues, suggesting that transduction of Cre alone into mESC does not impair self-renewal and pluripotency (Supplementary Fig S6B). In contrast, microinjection of GFP-Cre transduced cDKO-2 mESC failed to produce any midgestational embryos at all (Fig. 6A) from two separate experiments totaling 41 microinjections. Microinjection of control GFP transduced cDKO-2 mESC reproducibly produced embryos an average of 40% of the time, demonstrating the intrinsic ability of cDKO-2 mESC, in the absence of Cre, to contribute normally to embryogenesis. Interestingly, chimeric embryos produced with GFP-Cre transduced cDKO-3 mESC exhibited only about an one third reduction in embryo numbers at midgestation relative to cDKO-3 transduced with GFP control virus, however none of the GFP-Cre cDKO-3 chimeric embryos were morphologically normal (Fig. 6B). Chimeric embryos produced with GFP-Cre transduced cDKO-3 mESC exhibited strongly impaired development overall and an apparent reduced contribution of GFP+mESC to embryonic tissues (Supplementary Fig. S6C).

4. Discussion

Recent studies suggest that *myc* genes are critical for the acquisition (iPS cells) and maintenance (mESC and other stem cells) of stem cell properties, but their potential endogenous functions in self-renewal and pluripotency have not been clearly defined. The

possible role of *myc* genes in early embryogenesis has also been a longstanding open question. Here, we find that endogenous *myc* genes maintain ESC self-renewal and pluripotency, and are essential for early embryogenesis.

We observed pronounced upregulation of a variety of differentiation markers in the *myc* DKO mESC at a point where we did not observe significant loss in mRNA levels of transcriptional pluripotency markers such as *Oct4* and *Nanog.* The majority of *myc*-deficient cells expressed markers of early endoderm and mesoderm differentiation (GATA6 and Bmp4). About 20% of the population of *myc*-depleted cells expressed neuroectodermal marker Ngn3. However, there was no detectable expression of the more advanced differentiation markers such as AFP and SMA, and only about 5% of cells expressed TUJ1, a marker of more advanced neuronal differentiation. Since GATA6 precedes AFP expression in some endodermal cell types (Morrisey et al., 1998), and Bmp4 is expressed in early differentiating cells, compared to SMA, which is expressed in more differentiated smooth muscle cells, *myc*-depleted ES cultures thus mostly contain immature endo- and mesodermal precursors rather than fully differentiated cell types. The small population of more fully differentiated cells of neuronal lineage is interesting and suggests that in the absence of Myc, neuronal lineage progression may be unblocked more fully than other types of differentiation.

Myc-deficient mESC thus do not necessarily undergo terminal stages of differentiation to give rise to the fully differentiated progeny, but rather differentiate largely into early progenitor-like cells. Down-regulation of the main pluripotency markers is gradual upon differentiation, and it is likely that in the initial steps of differentiation, pluripotency factors are co-expressed with differentiation markers. Indeed, single-cell transcript analysis of hESC has demonstrated the persistence of pluripotency transcripts in differentiating cells, where multiple markers of differentiation and pluripotency are co-expressed at the single-cell level (Gibson et al., 2009). Together these data suggest that a predominant function for Myc in mESC pluripotency is potent suppression of early stages of differentiation. This notion is consistent with the finding that c-*myc* does not greatly contribute to the activation of pluripotency regulators in reprogrammed cells (Sridharan et al., 2009). However, we did observe modestly decreased protein levels of Sox2 and Oct4 upon *myc* disruption, which could be functionally important. This observation also suggests that *myc* can regulate ESC pluripotency not only through direct regulation of gene expression, but also through posttranscriptional events.

Additional evidence for a role of Myc in the repression of differentiation comes from observations that *myc* expression is normally downregulated upon induced differentiation of ESC (Sumi et al., 2007), and that overexpression of *myc* inhibits differentiation of a variety of cell types including hematopoietic and neuronal cells (Amanullah et al., 2000; Su et al., 2006). In addition, recent studies of the reprogramming factors involved in the induction of pluripotency have demonstrated that Myc proteins promote the induction of ESC gene expression programming in part by silencing of the somatic cell expression program (Sridharan et al., 2009). The comparison of gene expression programs operative in ESC and adult tissue stem cells has revealed two predominant gene modules that distinguish ESC and adult tissue stem cells (Wong et al., 2008). The adult tissue stem program has been reported to be downregulated by Myc (Wong et al., 2008). Interestingly, in our study, 55 genes that were up-regulated in both cDKO cell lines belong to the adult tissue stem module. The loss of *myc*, thus, might lead to ESC differentiation towards adult tissue stem cells, reflecting a loss of pluripotency.

In addition to potential direct suppression of expression of differentiation-associated genes, perhaps via Miz-1 (Wu et al., 2003), Myc activates expression of many genes in mESC.

While some activated target genes may in turn suppress differentiation, others likely act to directly maintain self-renewal, including cell cycle and chromatin regulatory genes. The loss of proliferation in the form of cell cycle disruption likely also plays some role in inducing spontaneous differentiation. Importantly, previous work has suggested a key effector role for c-Myc in LIF/STAT3 signaling in mESC since overexpressed c-Myc confers LIF-independence (Cartwright et al., 2005). Our finding that Myc is required for maintaining normal endogenous LIF expression in mESC fits with these previous studies but also suggests a complex positive feedback loop exists between Myc and LIF. Besides LIF, the other genes downregulated by loss of Myc included those involved in multiple aspects of cellular metabolism suggesting a key role for Myc in maintaining a highly active metabolic state that may be essential for mESC self-renewal, which we found to be strictly Myc-dependent. In addition to regulating expression of specific genes, Myc may have a more widespread role in regulating ESC chromatin suggested by studies in somatic stem and tumor cells (Martinato et al., 2008; Cotterman et al., 2008; Knoepfler et al., 2006; Guccione et al., 2006). By modulating the balance of global euchromatin and heterochromatin in ESC, Myc might, therefore, make the epigenome competent for the action of pluripotency factors, which could in turn activate pluripotency and self-renewal programs as well as block differentiation.

In our study we observed a significant ES cell growth inhibition, which, in addition to elevated levels of spontaneous differentiation, could also be attributed to Myc's ability to control cell cycle progression, cell survival, and general metabolism. Overexpression of Myc or its depletion leads to increased levels of apoptosis, suggesting that tight control of *myc* expression is obligatory for cell survival (Wang et al., 2008; Sumi et al., 2007; Wang et al., 2008). Consistent with this notion, our study has shown that loss of *myc* leads to a significant decrease in the population of cells in S phase with concomitant increase of the G_0/G_1 and the G_2/M cell populations as well as to elevated levels of apoptosis with concomitant increase in expression of cell cycle inhibitors including *gadd45* and *p57*, as well as genes involved in apoptosis in cDKO mES cells. Interestingly, although there is a considerable decrease in the population of cells in S phase, and *myc* depletion leads to a significant disruption of ES colony growth, approximately 50% of DKO cells are still in S phase. One of the possible explanations is that Myc depletion triggers a differentiation program in a subset of ES cells each time they are out of S phase, however it is unlikely that DKO cells fully undergo terminal differentiation, which is associated with the entry into G_0 phase of the cell cycle. *myc*-deficient ES cells might give rise to a variety of early progenitor-like partially differentiated cells, which grow more slowly and do not readily form colonies, but are likely still cycling, although their cell cycles are significantly slower than that of undifferentiated ESC with slower transit through all phases of the cell cycle. Cell cycle heterogeneity of partially differentiated cells thus might reconcile observed numbers of cells in S phase and significant colony growth disruption or delay. In addition to the cell cycle, the mechanisms by which *myc* maintains ESC self-renewal and pluripotency also likely include regulation of microRNAs (miRNA), the noncoding RNAs, which are known to have diverse roles including the regulation of cellular differentiation, proliferation, and apoptosis (Ambros, 2004). In ESC, miRNAs control cell cycle progression, differentiation, and lineage determination including hematopoietic and neural differentiation (Chen et al., 2004; Krichevsky et al., 2006; Ivey et al., 2008; Wang et al., 2008; Wang and Blelloch, 2009). Indeed, Dicer-null ESC that lack most mature miRNAs cannot differentiate into most lineages (Murchison et al., 2005). Recent data suggest that Myc regulates miRNA expression in a widespread fashion (Chang et al., 2008) and that Myc influences mESC biology at least in part through regulation of miRNA (Lin et al., 2009).

The role of *myc* genes in early murine embryogenesis has been an open question for more than two decades. While c- and N-*myc* constitutive knockouts are lethal around

midgestation, it has been unclear what role, if any, these genes played in early development. For the first time, our data clearly demonstrate that the early embryo is strongly *myc*-dependent. We have observed some differences between the chimeric embryos produced by the injections of cDKO-2 (unviable embryos) and cDKO-3 (developmentally abnormal embryos) cell lines, which could be explained by the differences in chimeric contributions between these cell lines. We hypothesize that cDKO-2 injected chimeric embryos have a more severe phenotype (inviable embryos) due to a higher degree of incorporation of this cell line into chimerae, compared to the cDKO-3 cell line in which chimeric contribution is high enough to cause developmental abnormalities, but not high enough to lead to an early embryonic lethality. High-contribution chimeras produced with mutant ES cells tend to display similar defects as homozygous mutant embryos (Douglas et al., 2001; Chamberlain et al., 2008). Chimeric embryos that were almost fully composed of c-Myc$^{-/-}$ ES cells exhibited developmental abnormalities by E10–E11, as reported for c-Myc$^{-/-}$ embryos, whereas chimeras with fewer incorporated c-Myc$^{-/-}$ ES cells developed normally (Douglas et al., 2001). Another possibility is that the cDKO-2 cell line had a more complete loss of *myc* expression prior to microinjection or more persistent Cre activity after injection, compared to cDKO-3 cell line. Importantly, the differences between cDKO-2 and cDKO-3 ES cell line survival are much less prominent in cell culture, compared to the chimeric embryo assays. This could be explained by the fact that in the embryo (in vivo), there are likely more strict requirements for Myc function in highly pluripotent stem cells, whereas in vitro, in the presence of growth factors and feeder layers, cell lines can more readily survive and perhaps proliferate modestly even with lowered Myc. In addition to embryogenesis, our results also have implications for the mechanisms by which Myc causes cancer, supporting the notion that Myc “locks in” an aberrant pluripotent state rendering tumors resistant to differentiation. This notion is also supported by the recent finding that N-Myc regulates expression of pluripotency genes in neuroblastoma (Cotterman and Knoepfler, 2009).

Our data also suggest that c- and N-*myc* can partially compensate for each other’s loss or are functionally redundant in ESC and the early embryo. The midgestational lethality of either single constitutive *myc* knockout reflects the incomplete nature of this compensation or redundancy. Intriguingly, through a placental rescue approach, the function of c-*myc* during development was recently defined to be predominantly to drive hematopoiesis (Dubois et al., 2008). This finding supports a model in which N-*myc* plays the more central role in self-renewal and pluripotency of the early embryo itself.

5. Conclusion

Taken together, our findings indicate that self-renewal and pluripotency of mESC as well as pluripotent cells of the early embryo are critically dependent upon *myc* expression. These findings have important implications for ES and iPS cell biology and regulation of early embryogenesis, but also for tumor stem cells and tumorigenesis. Additional insight into Myc function in highly pluripotent cells and its relevance to cancer await further study, but we propose a model in which Myc directs ESC biology and iPS cell formation through regulation of protein coding and miRNA gene expression to orchestrate general metabolism, pluripotency-related cell cycle machinery, and self-renewal. Myc may also promote a generally euchromatic state important for pluripotency. During tumorigenesis these functions are “locked in” promoting the transformation of normal stem cells into tumor stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.diff.2010.05.001.

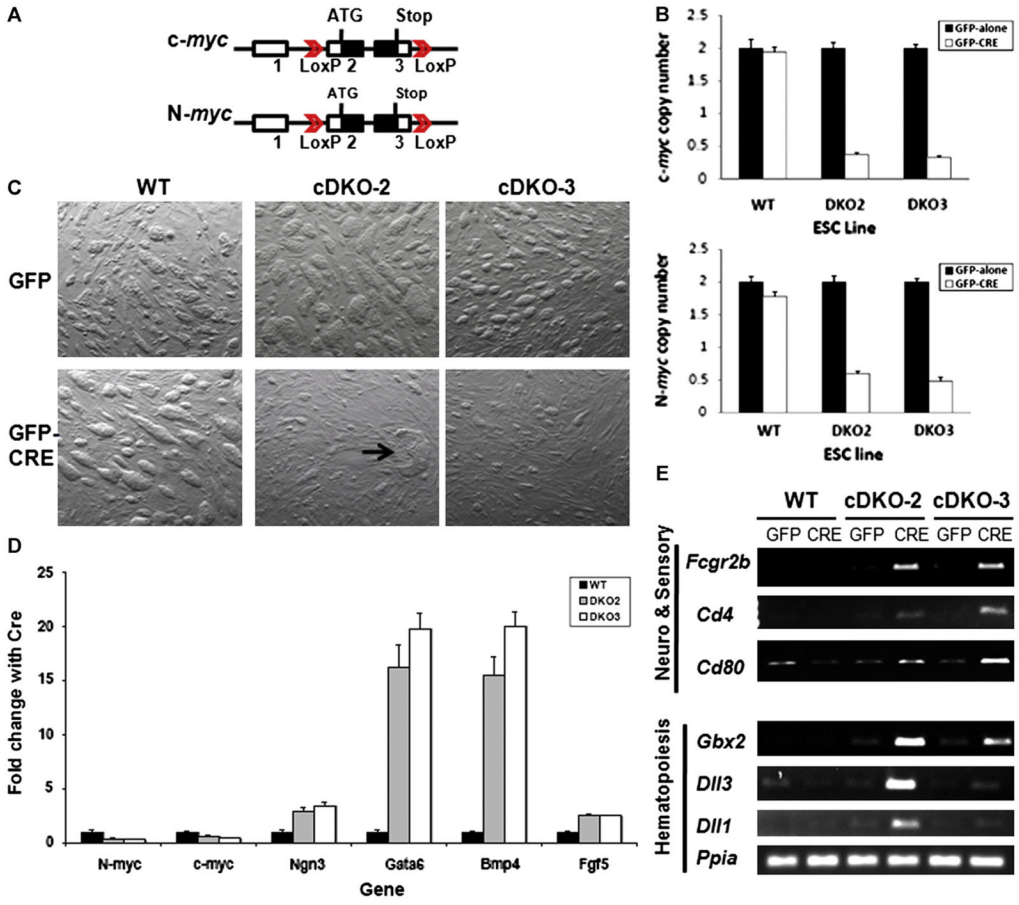

Fig. 1.

Loss of c- and N-*myc* disrupts mESC growth and triggers lineage commitment. (A) Schematic of the floxed c- and N-*myc* loci. (B) Detection of c- and N-*myc* gene copy number changes by real-time qPCR. Gene copy numbers for c- and N-*myc* were normalized using two reference genes, β-actin and Nat1. Error bars are standard deviations. Decreases in c- and N-*myc* copy numbers had p values of 8.3×10^{-7} and 7.2×10^{-7}, respectively, in cDKO2, and p values of 1.6×10^{-7} and 2.4×10^{-5} in cDKO3, respectively. (C) Phase contrast image of mESC lines in the presence or absence of CRE expression. Arrow denotes differentiated colony. GFP and GFP-Cre denoted cell cultures transduced with MSCV ires-GFP and MSCV Cre-ires-GFP retroviruses, respectively. (D) Real-time qRT-PCR of c-*myc*, N-*myc*, and a series of different lineage-specific marker genes in WT and DKO mESC lines. Levels of specific mRNAs measured by qRT-PCR were normalized to the levels of the loading control *Nat1*. Error bars are standard deviations. Changes in relative expression of N-myc, c-myc, Ngn3, Gata6, Bmp4, and Fgf5 had p values of 7×10^{-4}, 2×10^{-4}, 1×10^{-3}, 8.6×10^{-5}, 9×10^{-4}, and 9.6×10^{-4}, respectively, in cDKO2 and p values of 8.7×10^{-5}, 1.4×10^{-5}, 2.7×10^{-4}, 3.4×10^{-4}, 5.1×10^{-4}, and 1.3×10^{-3}, respectively, in cDKO3. (E) RT-PCR of hematopoietic, neural and sensory organ differentiation markers in mESC of the indicated genotype.

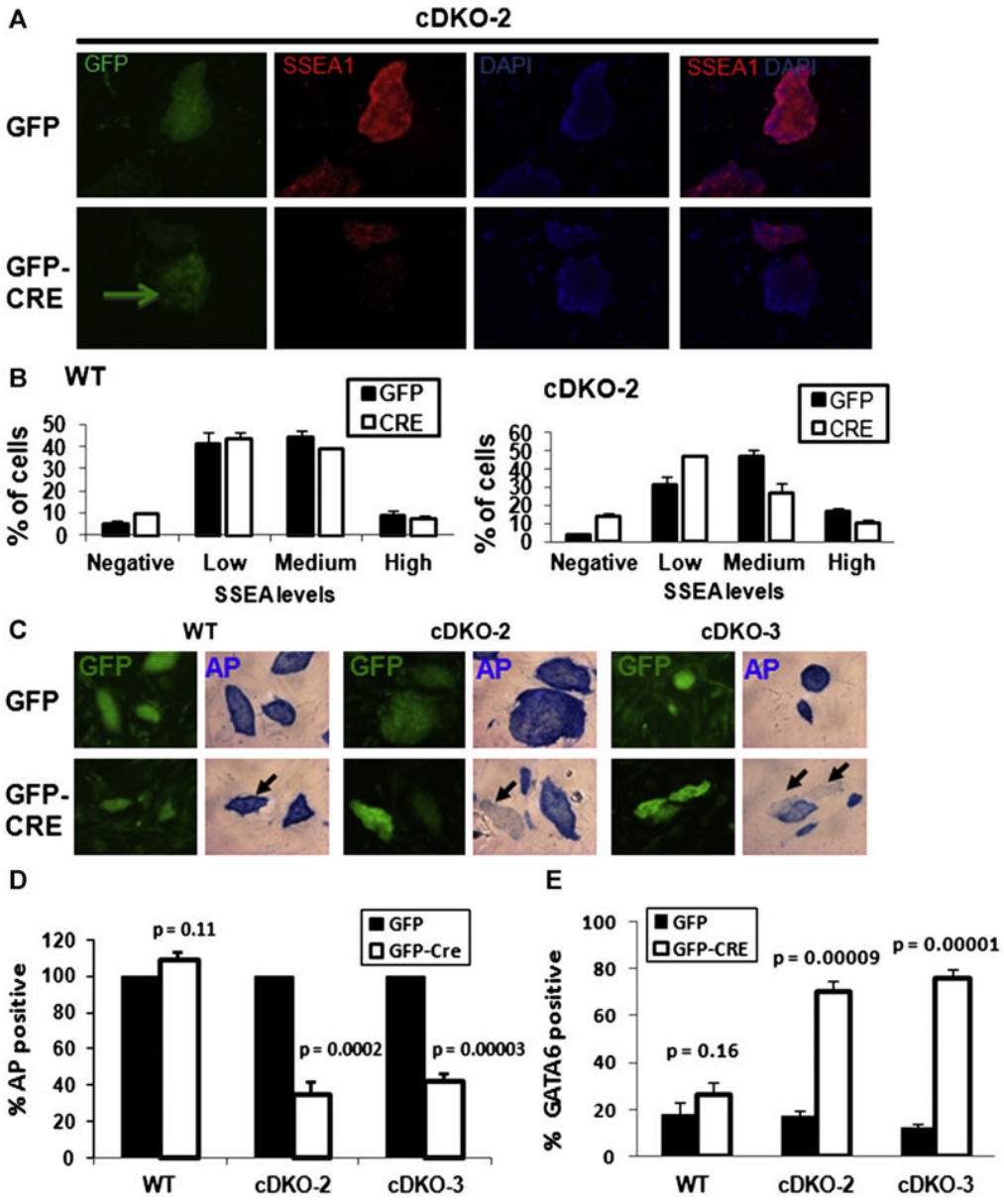

Fig. 2.

myc depletion disrupts mESC pluripotency and self-renewal. (A) Immunofluorescent staining for SSEA-1 (red) and DAPI (blue) in the cDKO-2 mESC line. Arrow marks a representative GFP+colony, negative for SSEA-1. (B) Flow cytometric analysis of SSEA-1 expression in DKO-2 mESC line. mESC were analyzed by FACS for SSEA-1 levels with levels gated and defined as follows: negative (equal to or below levels of expression of isotype specific negative control), low (bottom third), medium (middle third), and high (top third) of SSEA-1 levels. Error bars are standard deviations. *N*=3. Decreases in high and medium SSEA-1 staining and increases in low and negative SSEA-1 staining had *p* values of 0.002, 0.005, 0.002, and 0.01, respectively, in cDKO2 and *p* values of 0.01, 0.002, 0.004, and 0.006 in cDKO3, respectively. (C) Fluorescent and phase contrast images of WT and DKO mESC stained for AP. Arrows denote AP staining in representative GFP-Cre positive colonies. (D) Percentage of self-renewing colonies of WT and DKO mESC lines calculated

after alkaline phosphatase assays. Percentage of AP positive colonies in GFP only transduced mESC lines was defined as 100%. The error bars are SD and the data is the mean from three biological replicates (n=3). (E) Percentage of GATA6 positive cells out of GFP positive mESC (GFP alone or GFP-CRE) quantified by immunofluorescent staining for GATA6. The error bars are SD and the data is the mean from three biological replicates (n=3). P values were calculated by two-tail t-test assuming equal variances throughout this figure.

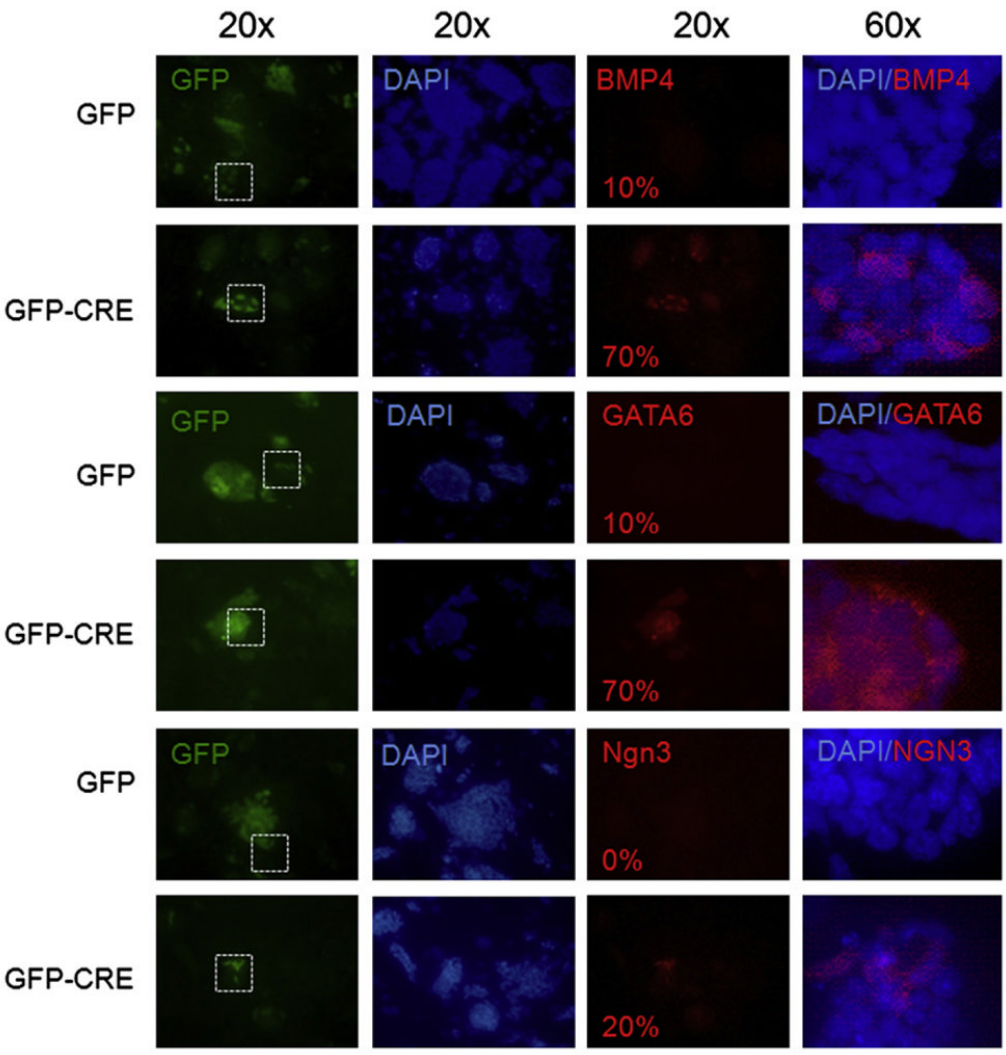

Fig. 3.

myc knockdown stimulates the expression of early differentiation markers. Immunofluorescent staining for the expression of Bmp4, GATA6, and Ngn3 in GFP control and GFP-CRE transduced DKO mESC colonies. Numbers in the bottom left corner indicate the percentage of cells expressing Bmp4, GATA6 or Ngn3 differentiation markers out of GFP positive mESC (GFP alone or GFP CRE). Images taken at 20× magnification show the expression of differentiation markers in mESC colonies, and images taken at 60× magnification show the expression of differentiation markers in individual cells within mESC colonies. White square defines region of 20× field shown in 60× panel.

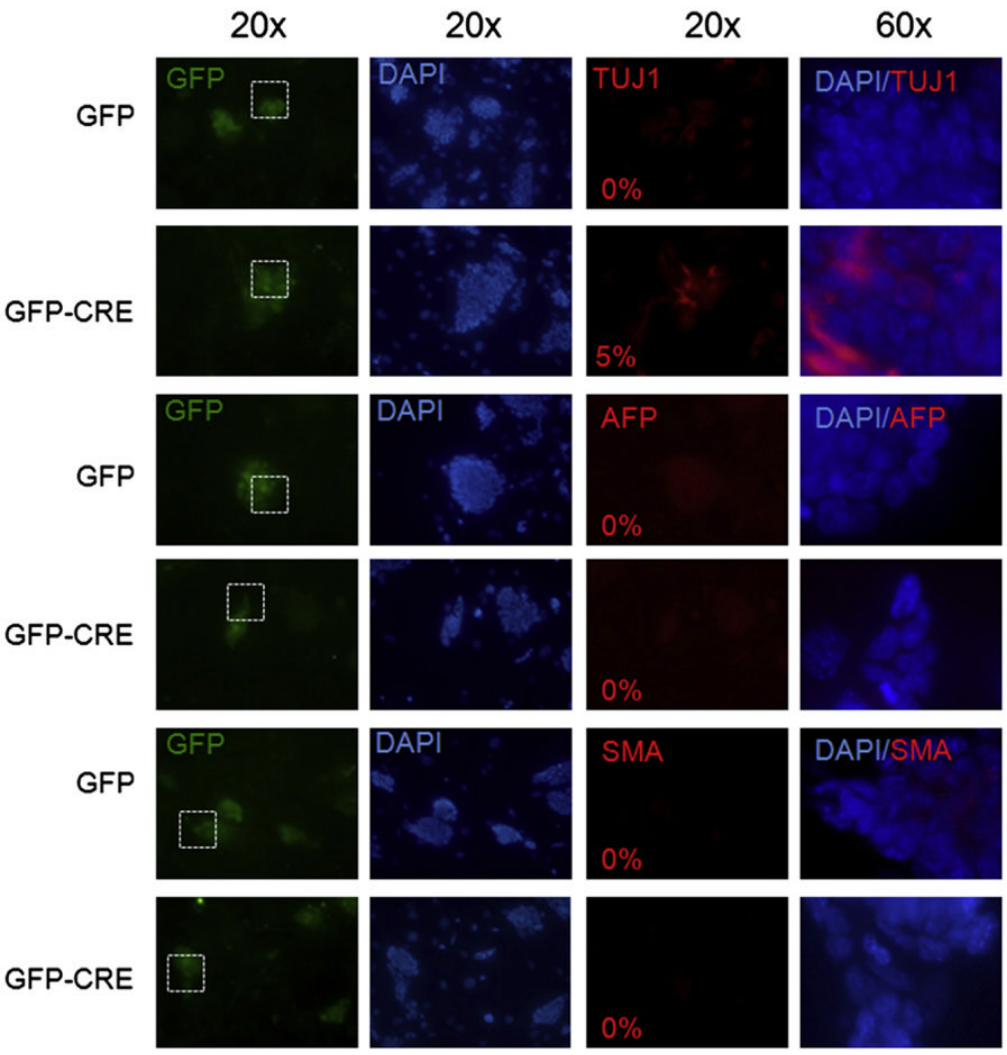

Fig. 4.

myc-deficient cells do not exhibit widespread expression of late differentiation markers. Immunofluorescent staining for the expression of TUJ1, SMA, and AFP in GFP alone and GFP-CRE transduced DKO mESC colonies. Numbers in the bottom left corner indicate the percentage of cells expressing TUJ1, SMA, or AFP differentiation markers out of GFP positive mESC (GFP alone or GFP CRE). Images taken at 20× magnification show the expression of differentiation markers in mESC colonies, and images taken at 60× magnification show the expression of differentiation markers in individual cells within mESC colonies. White square defines region of 20× field shown in 60× panel.

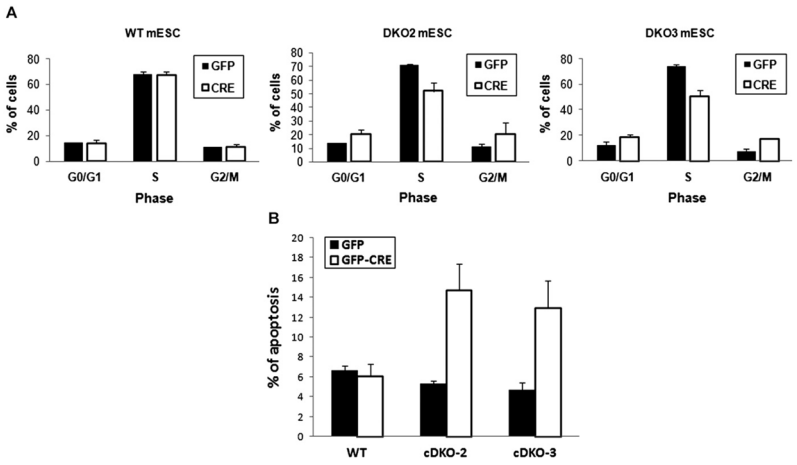

Fig. 5.

Myc is required for cell cycle progression and cell survival of mESC. (A) Cell cycle profiles of WT and cDKO mESC lines were obtained by measuring bromodeoxyuridine (BrdU) incorporation coupled to the staining of DNA content with 7-amino-actinomycin D (7-AAD). Changes in G_0/G_1, S, and G_2/M phases had *p* values of 0.02, 0.003, and 0.08 for cDKO2 and *p* values of 0.04, 0.0003, and 0.007 in cDKO3, respectively. (B) Percentage of apoptotic cells in GFP positive populations in mESC lines was measured by Annexin-V staining assay. The data are the means of three biological replicates (*n*=3) and error bars are standard deviations. Increases in apoptosis had *p* values of 0.004 and 0.006 in cDKO2 and cDKO3, respectively.

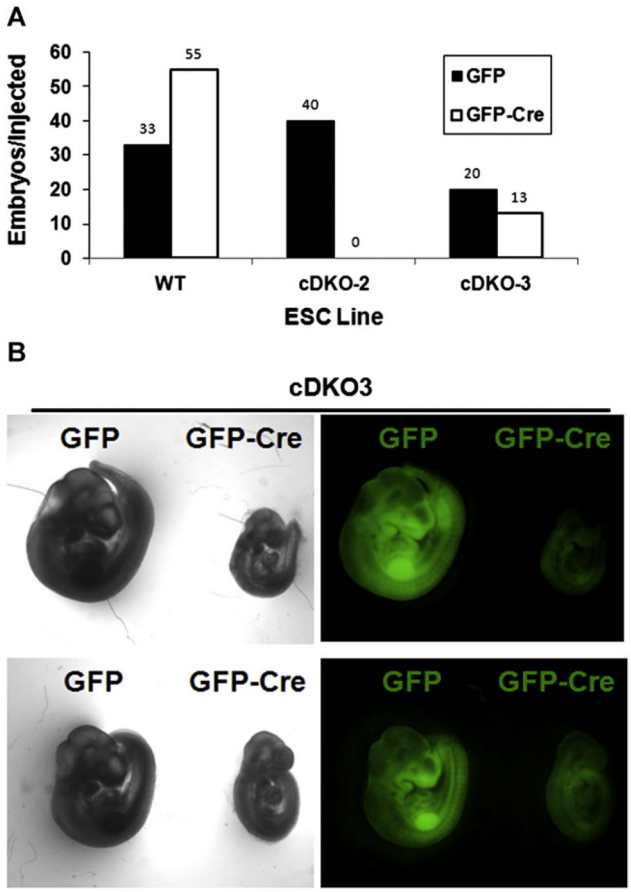

Fig. 6.

Myc is essential for early embryogenesis. (A) Mean percentage of midgestational chimeric embryos recovered after 8–9 days postinjection in two independent microinjection experiments. The graph represents the percentage of chimeric embryos which were recovered compared to the total number of injected embryos for each cell type (defined as 100%). (B) Representative phase contrast and fluorescence microscopy images of chimeric embryos produced with cDKO-3 mESC transduced with GFP or GFP-Cre virus.