LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism

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Accepted 22 December 2004

Development 132, 885-896
Published by The Company of Biologists 2005
doi:10.1242/dev.01670

Summary

Murine ES cells can be maintained as a pluripotent, self-renewing population by LIF/STAT3-dependent signaling. The downstream effectors of this pathway have not been previously defined. In this report, we identify a key target of the LIF self-renewal pathway by showing that STAT3 directly regulates the expression of the Myc transcription factor. 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), triggering its GSK3β dependent degradation. Maintained expression of stable Myc (T58A) renders self-renewal and maintenance of pluripotency independent of LIF. By contrast, expression of a dominant negative form of Myc antagonizes self-renewal and promotes differentiation. Transcriptional control by STAT3 and suppression of T58 phosphorylation are crucial for regulation of Myc activity in ES cells and therefore in promoting self-renewal. Together, our results establish a mechanism for how LIF and STAT3 regulate ES cell self-renewal and pluripotency.

Key words: ES cells, Self-renewal, Myc, Pluripotency

Introduction

In the presence of IL6 family members such as leukemia inhibitory factor (LIF), murine embryonic stem (ES) cells can be maintained indefinitely in a self-renewing state that closely resembles the pluripotent cells of the inner cell mass (ICM). LIF signaling in murine ES cells is initiated through dimerization of the cytokine receptors LIF-R and gp130 following their engagement by cytokine. This signals the activation of Jak family non-receptor tyrosine kinases which phosphorylate LIF-R and gp130 on tyrosine residues (Boulton et al., 1994). STAT3 is then recruited to the receptor complex where it is phosphorylated by Jak, resulting in its subsequent dimerization, nuclear translocation and target gene activation (Darnell, 1997). Although ectopic STAT3 activity promotes stem cell self-renewal and maintenance of pluripotency in the absence of LIF (Matsuda et al., 1999), inactivation of STAT3 function in LIF-maintained ES cells promotes spontaneous differentiation (Niwa et al., 1998). These lines of evidence establish STAT3 as an essential component of the LIF-dependent self-renewal pathway. With the exception of STAT3, however, downstream effectors of the LIF-dependent self-renewal pathway have not been defined in ES cells.

Murine ES cells are typically cultured in the presence of fetal calf serum (FCS) or a defined cocktail of supplements that in conjunction with LIF, support proliferation and stem cell maintenance. Although LIF has a well-characterized pro-maintenance function, other factors present in serum have generally been overlooked in terms of performing a specific role in promoting self-renewal. Ying and co-workers (Ying et al., 2003) recently reported, however, that factors in FCS such as bone morphogenic proteins (BMPs) may work in collaboration with LIF to promote self-renewal. In the presence of LIF, BMPs antagonize neural differentiation (Ying et al., 2003) and, in parallel, promote self-renewal through suppression of ERK/MAPK signaling (Qi et al., 2004). Maintenance of a stable stem cell state therefore appears to require multiple inputs that impose lineage-specific differentiation blocks (Chambers et al., 2003; Mitsui et al., 2003; Ying et al., 2003).

Although LIF/STAT3 signaling is crucial for murine ES cell maintenance, this pathway does not appear to have a role in human ES cell self-renewal (Humphrey et al., 2004), indicating the existence of alternate self-renewal mechanisms. Sato and co-workers (Sato et al., 2004) recently defined a role for Wnt-dependent signaling in self-renewal of human and murine ES cells that functions independently of LIF and STAT3. Moreover, suppression of GSK3β, an antagonist of Wnt signaling, is sufficient to maintain self-renewal and pluripotency of human and murine ES cells in the absence of LIF and Wnt (Sato et al., 2004). These observations signify a common mechanism of self-renewal that may be further applicable to adult stem cell populations that require Wnt-dependent signaling (Reya et al., 2003).

Although LIF and Wnt promote self-renewal by activation of separate signaling pathways, we reasoned that they would converge on a common target(s). We hypothesized that Myc could be a common effector on which these signals converge because the Myc gene is a transcriptional target of STAT3 in a number of biological contexts (Kiuchi et al., 1999; Shirogane et al., 1999; Bowman et al., 2001), and signals transduced by Wnt can activate the Myc transcription through a β-catenin/TCF-dependent mechanism (He et al., 1998). Myc

belongs to a family of helix-loop-helix/leucine zipper transcription factors and together with its obligatory binding partner, Max, performs roles in control of cell proliferation, transformation, growth, differentiation and apoptosis. A potential role for Myc in ES cell maintenance is suggested by two reports. First, expression of an RLF/L-myc minigene that frequently arises from a chromosomal translocation event in human small lung carcinomas, delays ES cell differentiation and interferes with early embryonic development (MacLean-Hunter et al., 1994). Second, elevated Myc activity is able to block the differentiation of multiple cell lineages (Selvakumaran et al., 1996; Canelles et al., 1997; Pelengaris et al., 1999; Schreiner et al., 2001; Knoepfler et al., 2002). These lines of evidence prompted us to investigate whether Myc plays a role in ES cell self-renewal downstream of LIF and/or Wnt. In this report, we show that elevated Myc activity is required for ES cell maintenance and that Myc is a key effector of the LIF/STAT3 self-renewal pathway. Our data indicate that signals transduced by LIF and possibly Wnt, converge on Myc to maintain ES cell identity.

Materials and methods

Cell culture and transfection

D3 (Doetschman et al., 1985) and R1 EGFP (Hadjantonakis et al., 1998) ES cells were maintained on gelatinised plates (0.2% gelatin in PBS; 1.6×10^5 cells/cm^2) in ES complete (ESC) medium consisting of high glucose DMEM supplemented with 10% FCS, 1 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 10% knockout SR (InVitrogen) at 37°C under 10% CO_2, supplemented with 1×10^3 units/ml LIF (ESGRO, Chemicon). Experiments were first performed in the D3 line and then confirmed in the R1 line. Alternatively, cells were grown in ESC media conditioned by L-M tk– cells (ATCC, CRL-2648) or by Wnt3a secreting L-M tk– cells (ATCC, CRL-2647), prepared as per the suppliers recommendations (see Sato et al., 2004). Cells were passaged every 3 days using trypsin-EDTA (Invitrogen) and plated at 1.6×10^5 cells/cm^2. For the generation of stable transfectants, 2×10^7 cells were re-suspended in 1 ml PBS and electroporated with 20 μg linearized plasmid DNA (200 V, 500 μF). After 24 hours recovery, transfected cells were selected in puromycin (1 μg/ml) or neomycin (200 μg/ml) for between 7 and 10 days then clonally expanded in the presence of drug selection. Four independent clonal cell lines were typically selected for analysis to determine that the properties of each cell line set was consistent.

ES cell maintenance, differentiation assays and colony forming assays

For long-term maintenance, mycER and myc^{T58A}ER cells were grown in ES complete media supplemented with 4OHT (100 nM or 2-10 nM, respectively) and re-fed every second day. For differentiation of ES cells as embryoid bodies (EB), cells were trypsinized and replated on bacteriological dishes at 1.6×10^5 cells/cm^2 and grown in ESC media (–LIF) plus or minus 4OHT to allow for EB formation. Cells were re-fed every second day and expanded after 4 days of culture. mycER cells were passaged by limited trypsinization, avoiding generation of single cell suspensions. AP activity was assayed with Alkaline Phosphatase Substrate Kit I (Vector Laboratories) by scoring 100 individual ES cell colonies. AP-positive colonies were uniform and dome-shaped in morphology, and consisted of densely packed AP-positive cells. ES colonies were classified as being negative in the absence of AP staining or when a mixture of stained and unstained cells were observed with a corresponding flattened, non-uniform colony morphology. Colony forming assays were performed by plating 500 cells at clonal density in ESC medium, followed by scoring colonies for AP activity after 5 days.

Antibodies, flow cytometry and immunoblot analysis

The following antibodies were used: phospho T58 Myc (Cell Signaling Technology), anti-Myc (N-262; Santa Cruz), anti-Oct4 (N-19; Santa Cruz), anti-tubulin (Serotech), anti-HDAC1 (Zymed), anti-Cdk2 (M2; Santa Cruz) and anti-GSK3β (Transduction Laboratories). The anti-HDAC1 antibody detects both HDAC1 and HDAC2 and accordingly, a doublet is seen by immunblot analysis. For flow cytometry, ES cells (2×10^6) were washed with 1×PBS and fixed in 2% paraformaldehyde in 1×PBS for 10 minutes at room temperature. Cells were then washed (1×PBS), incubated with anti-SSEA1 mouse monoclonal antibody (Chemicon, 1:100) at 4°C for 30 minutes, washed twice and resuspended in anti-mouse Alexa-488 secondary antibody (1:1,000; Molecular Probes) in 1% BSA/PBS at 4°C for 30 minutes. Finally, cells were washed twice, resuspended in 1% BSA/1×PBS and analysis performed using a Beckman Coulter FC500 flow cytometer.

Cells were lysed by incubating for 1 hour on ice in lysis buffer consisting of 50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM EGTA, 250 mM NaCl, 10% glycerol, 0.1% Tween-20, 10 μg/ml TPCK, 50 μg/ml TLCK, 170 μg/ml PMSF, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin and phosphatase inhibitor cocktail II (Calbiochem) with occasional vortexing/pipetting. Whole-cell extracts (20-40 μg total protein) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (BioRad), blocked with 3% BSA in TBSE (Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA and 0.1% NP40) and probed with the indicated antibodies at 4°C.

GSK3β kinase assays

Cell lysate (300 μg protein at 1 mg/ml) in cell lysis buffer was tumbled with 3 μg of primary antibody for 3 hours at 4°C. Protein A Sepharose beads (30 μl) were added and tumbled for a further 1 hour at 4°C. Beads were washed three times with 1 ml of cold lysis buffer then once with 1 ml of kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA and protease inhibitor cocktail II, Calbiochem) and resuspended in 30 μl of kinase buffer with or without the addition of GSK3 inhibitor II (3 μM in ethanol, Calbiochem) or ethanol. Reactions were incubated at 30°C for 20 minutes with 10 μCi of [α-^{32}P] ATP and 5 μg of myelin basic protein (MBP, Upstate Biotech) at 30°C for 20 minutes. Reactions were terminated by the addition of 2×SDS load buffer then resolved by SDS PAGE. Kinase activities were quantitated by phosphorimager analysis.

Plasmids, northern blot, RT-PCR analysis and ChIP assays

Details of all plasmid constructs are available on request. Total RNA from ES cells was prepared with TRIzol Reagent (InVitrogen), Northern blot analysis and probe synthesis were as described previously (Stead et al., 2002). Primer sequences for RT-PCR analysis and PCR conditions were as described previously (Oka et al., 2002). Chromatin immunoprecipitation assays were performed essentially as described by Fernandez et al. (Fernandez et al., 2003). Sequences of primers used for RT-PCR and ChIP analysis are available on request.

Generation and analysis of chimeric mice

4OHT-maintained R1-EGFP/myc^{T58A}ER ES cells (129 background) were injected into blastocyst stage C57BL/6 embryos and reimplanted into pseudopregnant females as described by Hogan et al. (Hogan et al., 1994). Embryos were analyzed for GFP fluorescence of whole embryos (12.5 dpc) and by coat color of adult mice.

Results

Myc is a STAT3 target gene in ES cells

To understand more about the possible role of Myc in ES cell

self-renewal and differentiation, we initially characterized its regulation in D3 ES cells and during embryoid body (EB) differentiation. Formation of ES cell aggregates in suspension, in the absence of LIF, allows for the formation of EBs where differentiation proceeds into the three germ layers ectoderm, endoderm and mesoderm in a manner reminiscent of embryonic events associated with gastrulation. RT-PCR analysis showed that Myc transcripts were elevated in ES cells but declined by day 2 of EB differentiation. This closely paralleled the decline in mRNA levels of the ES/ICM cell marker Rex1, but preceded the appearance of mRNAs for the primitive ectoderm marker Fgf5, and the mesoderm marker brachyury (Fig. 1A). Myc mRNA levels therefore collapse soon after the inactivation of LIF/STAT3 signaling in ES cells.

To test the hypothesis that the Myc gene is a downstream effector of LIF/STAT3 signaling in ES cells, we tested whether it was a STAT3 target by chromatin immunoprecipitation (ChIP) analysis. $STAT3_{FLAG}$ was expressed from a CAG promoter because of its well established use in ES cell expression assays, having the advantage of modest expression levels (Matsuda et al., 1999; Niwa et al., 1998). FLAG epitope tagged STAT3 ($STAT3_{FLAG}$) was immunoprecipitated with crosslinked chromatin and its association with the Myc promoter evaluated by PCR analysis. The promoter site chosen for analysis consists of a conserved overlapping E2F/E-box element previously shown to be regulated by STAT3 in other cell types (Kiuchi et al., 1999). Specific co-precipitation between $STAT3_{FLAG}$ and the Myc promoter was seen in ES cells but this decreased during EB differentiation from day 1 onwards (Fig. 1B). This is consistent with the DNA-binding activity of STAT3 decreasing following cessation of LIF signaling (Niwa et al., 1988). To confirm that STAT3 binding was specific to the Myc gene, we show that STAT3 was not detected on the CDK1 promoter at any time point during the experiment. We conclude that the Myc gene in ES cells is a direct target of STAT3 in vivo.

Responsiveness of the Myc gene to LIF signaling and the direct recruitment of STAT3 to the Myc promoter suggested that STAT3 trans-activates the Myc gene in vivo. To address this, we evaluated the responsiveness of the endogenous Myc gene to activation by STAT3-ER which in the presence of 4-hydroxytamoxifen (4OHT), can maintain ES cells in the absence of LIF (Matsuda et al., 1999). By adding 4OHT to cultures, the labile form of ER fusions can be switched to a biologically active state by releasing them from chaperone proteins (see Eilers et al., 1989). In the case of nuclear factors, this involves their steroid-induced translocation to the nucleus. D3 ES cells were grown with LIF and then in the absence of LIF for 36 hours. 4OHT or LIF was then re-added to cultures for a further 24 hours, and Myc mRNA levels determined to evaluate the responsiveness of the endogenous Myc gene to activated STAT3 and restored LIF signaling. Following withdrawal of LIF, endogenous Myc mRNA levels decreased but this was reversed following readdition of LIF (Fig. 1C). Restoration of STAT3 signaling by addition of 4OHT (–LIF) had a similar effect within 24 hours (Fig. 1C), indicating that STAT3 trans-activates the Myc gene in ES cells in vivo. These results demonstrate that the Myc gene is a direct transcriptional target of LIF/STAT3 signaling in ES cells.

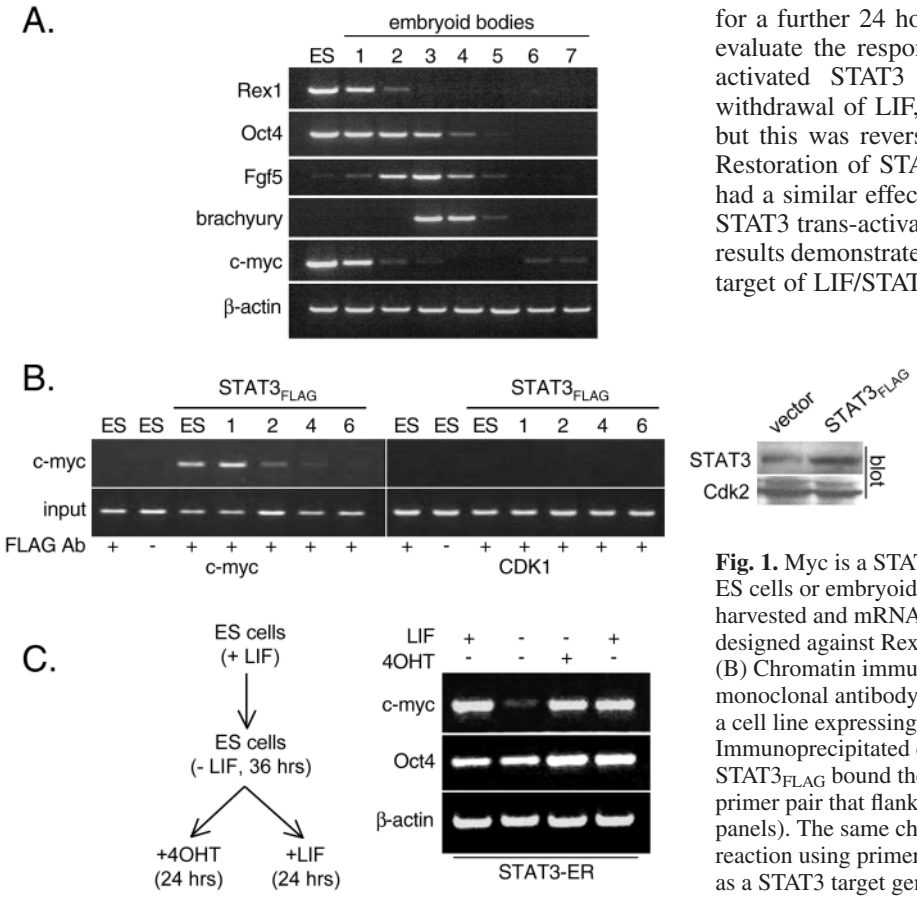

Fig. 1. Myc is a STAT3 target gene in ES cells. (A) LIF-maintained ES cells or embryoid bodies (–LIF) generated from ES cells were harvested and mRNA analysed by RT-PCR analysis using primers designed against Rex1, Oct4, Fgf5, brachyury, Myc and β-actin. (B) Chromatin immunoprecipitation (ChIP) analysis with a FLAG monoclonal antibody was performed on crosslinked chromatin from a cell line expressing FLAG-tagged STAT3 ($STAT3_{FLAG}$). Immunoprecipitated chromatin was then used to determine if $STAT3_{FLAG}$ bound the endogenous Myc promoter using a specific primer pair that flanks an E2F/E-box in the Myc P2 promoter (left panels). The same chromatin was used as a template in a PCR reaction using primer for the CDK1 promoter that is not implicated as a STAT3 target gene (right panel). Addition of FLAG antibody in ChIPs is as indicated (+). To control for the amount of total chromatin in the ChIP assay, equivalent amounts of total template ('input'), equivalent to 0.03% of total chromatin DNA used in ChIP assays, were used in a parallel PCR reaction using Myc or CDK1 specific primers. Immunoblot analysis: relative expression levels of total STAT3 in the transfected cell line is shown relative to endogenous STAT3 levels (vector alone transfectant). (C) STAT3 trans-activates the Myc gene in ES cells. LIF maintained STAT3-ER cells were deprived of LIF for 36 hours, stimulated with 4OHT (10 nM) or LIF (1×10^3 units /ml) and assayed 24 hours later by RT-PCR analysis.

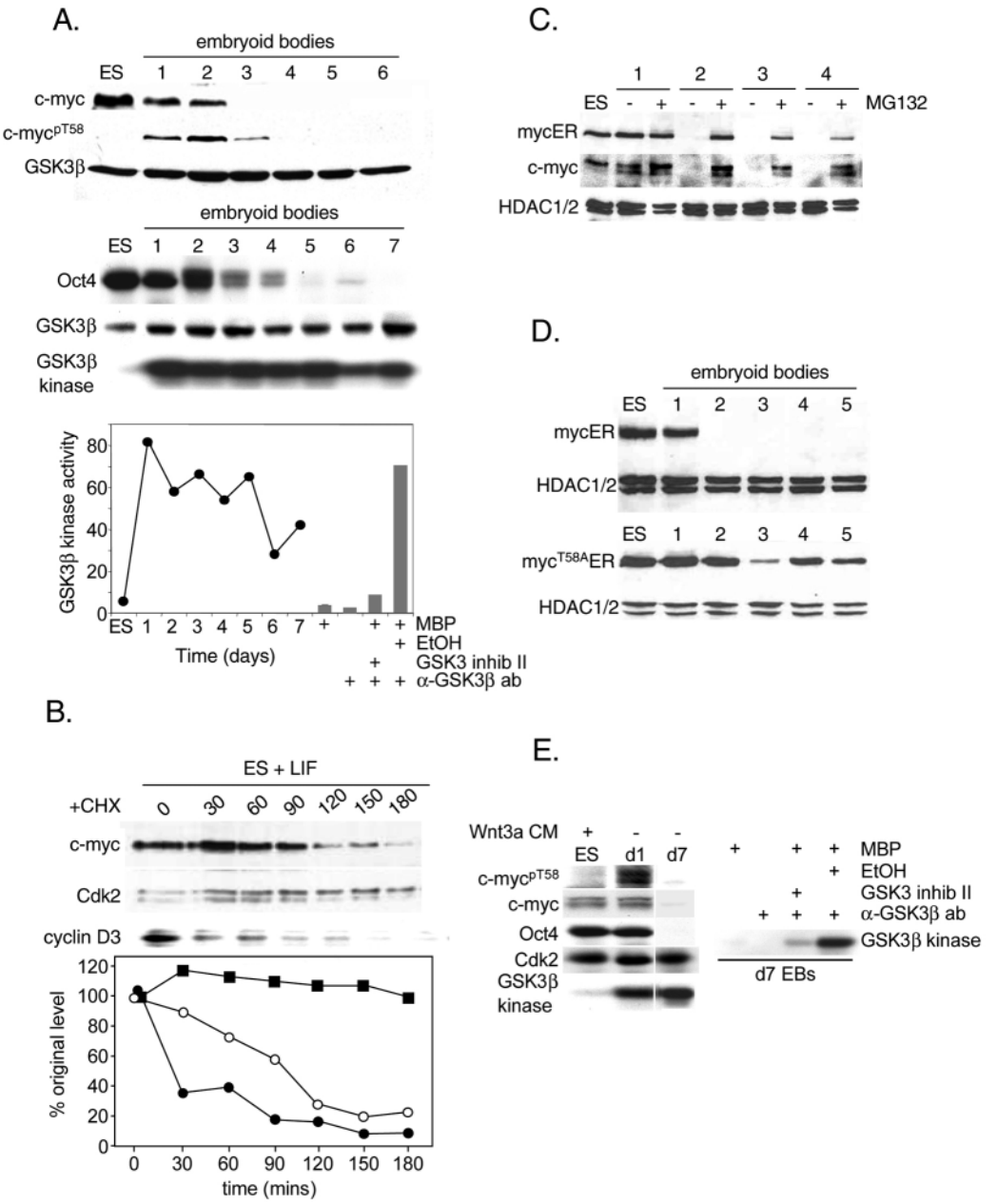

Fig. 2. Maintenance of Myc levels in ES cells requires suppression of T58 phosphorylation. (A) Top panel: whole cell lysates (40 μg total protein) from LIF maintained ES cells and embryoid bodies (–LIF; see A) were immunoblotted and probed with antibodies raised against Myc, phospho-T58 Myc (Myc^{pT58}) and GSK3β; middle panel, extracts as in top panel were probed with anti-Oct4, and GSK3β antibodies. GSK3β kinase activity at corresponding times is shown. Bottom panel: quantitation of GSK3β kinase activity in LIF-maintained ES cells and during differentiation was performed by phosphorimaging analysis and is depicted as a fold increase over initial levels in ES cells. Specificity of the assay was determined by immunoprecipitating GSK3β from day 6 EB cell lysates and performing kinase reactions in the presence of MBP, GSK3 II inhibitor, ethanol as indicated by +. The presence of the GSK3β antibody in the immunoprecipitation is as indicated (+). (B) ES cells maintained in LIF were treated with cycloheximide (+CHX, 10 μM) and at 30 minute intervals cells were harvested and whole cell lysates prepared. Following immunoblotting, extracts were probed with antibodies as indicated. Levels of Myc (white circles), Cdk2 (black squares) and cyclin D3 (black circles) are shown for each time point relative to pre-cycloheximide chase levels (t=0). (C) Whole-cell lysates were prepared from mycER LIF-maintained ES cells and day 1, day 2, day 3 and day 4 EBs (–LIF) that had been treated with proteosome inibitor MG132 (+, 5 μM) or ethanol alone (–) 3 hours prior to harvesting. After SDS PAGE and electroblotting onto a membrane, cell lysates (40 μg total protein) were then probed with antibodies raised against Myc and HDAC1/2. (D) Mutation of T58 (T58A) delays the downregulation of Myc protein following LIF withdrawal. mycER or myc^{T58A}ER levels were evaluated in LIF-maintained lines or in EBs generated by growth in the absence of LIF over 5 days (1-5) by immunoblot analysis using anti-Myc and anti-HDAC1 antibodies (loading control). (E) ES cells maintained in Wnt3a CM for five passages (15 days) were harvested or grown as EBs in the absence of CM for 1 or 7 days. Cell lysates were probed with antibodies as indicated and GSK3β kinase assays performed as described in A but on extracts made from day 7 EBs. The specificity of kinase assays was confirmed as described in A.

Maintenance of Myc levels in ES cells requires suppression of T58 phosphorylation

For Myc to be a regulator of self-renewal we predicted that its activity would rapidly decline following LIF withdrawal. To investigate this, we evaluated Myc protein levels in ES cells and during EB differentiation. Levels of Myc protein were elevated in LIF-maintained ES cells but declined markedly by day 1 of LIF withdrawal and even further over days 1-3 (Fig. 2A). The downregulation of Myc protein occurs well before Oct4 mRNA levels are extinguished and prior to the appearance of mRNAs for Fgf5 and brachyury (Fig. 1A). Downregulation of Myc protein is therefore associated with loss of LIF signaling and the early stages of differentiation.

Although changes in Myc mRNA could account for the collapse of Myc protein following LIF withdrawal, post-transcriptional mechanisms are also involved in regulating Myc protein levels. A key step in the mechanism regulating Myc degradation involves its GSK3β-dependent phosphorylation on threonine 58 (T58) (Sears et al., 2000; Gregory et al., 2003; Welcker et al., 2004; Yeh et al., 2004). To determine if T58 was phosphorylated in a manner consistent with it being targeted to initiate Myc proteolysis following removal of LIF, the same extracts were probed with a T58 phospho-specific antibody (Fig. 2A). The pT58 form of Myc was absent in ES cells but increased markedly upon LIF withdrawal with kinetics consistent with it being an initiating event in the degradation of Myc (Fig. 2A). As GSK3β is the principal regulator of T58 phosphorylation, we tested if it could account for phosphorylation of Myc. This was evaluated by immunoprecipitating GSK3β from whole cell lysates and then evaluating kinase activity through its ability to phosphorylate myelin basic protein (MBP) in vitro. In ES cells, GSK3β kinase activity was low but within 1 day of LIF withdrawal was induced ~15-fold (Fig. 2A). This could not be accounted for by changes in GSK3β protein levels as they remained relatively constant (Fig. 2A). The specificity of the assay was confirmed by demonstrating that the immunoprecipitated kinase activity from day 6 EBs was sensitive to a specific inhibitor of GSK3 (GSK inhibitor II; Fig. 2A). Changes in GSK3β activity did not correlate with the decline in Oct4 protein levels which occurred from day 2 onwards, but paralleled closely the phosphorylation of Myc on T58. These results support the hypothesis that GSK3β triggers Myc degradation through phosphorylation of T58 in ES cells.

As phosphorylation at T58 could not be detected in ES cell lysates, this suggested that signals involved in Myc degradation, such as that generated by GSK3β, were suppressed in ES cells. If so, we predicted that Myc would have enhanced stability compared with other cell types where its half-life ($t_{1/2}$) is ~20-30 minutes or less. Cycloheximide chase experiments in ES cells showed that in the presence of LIF, Myc has a half life of 105 minutes (Fig. 2B). Enhanced Myc stability is therefore an important determinant of Myc levels in ES cells. By comparison, the $t_{1/2}$ values of Cdk2 and cyclin D3 were over 180 minutes and 25 minutes, respectively. Parallel experiments showed that Myc $t_{1/2}$ in NIH 3T3 fibroblasts is less than 30 minutes (P.C. and S.D., unpublished).

To further demonstrate that increased turnover of Myc correlates with the activation of GSK3β and T58 phosphorylation, we differentiated ES cells as EBs and evaluated the stability of Myc in the presence or absence of proteosome inhibitor MG132. The cell line used also stably expressed Myc fused to the steroid-binding domain of the estrogen receptor, making it possible to evaluate the role of post-transcriptional control in Myc regulation, because it is expressed from the constitutive CAG promoter, which is active throughout differentiation in vitro and in all tissues in vivo (Okabe et al., 1997; Pratt et al., 2000). At day 2 after LIF withdrawal, levels of Myc and mycER collapsed but this was completely blocked by addition of MG132 (Fig. 2C). Besides indicating that the decline of Myc levels is proteosome dependent, these results show that post-transcriptional controls are crucial in determining Myc levels during differentiation. This point is underscored by the parallel collapse of mycER levels, even though transcription of the fusion protein was driven by the constitutively active CAG promoter (Okabe et al., 1997; Pratt et al., 2000). Recovery of detectable Myc in day 2, day 3 and day 4 EBs treated with MG132 indicates that residual Myc transcription generates a labile pool of Myc protein that is rapidly degraded and not readily detected by immunoblot analysis.

To confirm that downregulation of Myc protein levels following LIF withdrawal involved a T58-dependent mechanism, we evaluated levels of a Myc estrogen receptor (ER) fusion protein and the corresponding myc^{T58A}ER fusion driven by the CAG promoter in differentiating EBs. A comparison of cell lines expressing both forms of mycER fusion, in the presence of 4OHT, revealed that the T58A mutant persisted for at least 5 days after LIF withdrawal compared with levels of the T58 form, which had collapsed from day 1 (Fig. 2D). These observations indicate that downregulation of Myc during differentiation occurs through a T58-dependent mechanism.

As Wnt3a activity can contribute to self-renewal of ES cells (Sato et al., 2004), we asked if Myc was elevated under conditions of Wnt3a-dependent maintenance and if it was phosphorylated on T58 after withdrawal of the Wnt3a signal. To test this idea, we used L-fibroblast conditioned media (CM) containing secreted Wnt3a as a source of Wnt (see Sato et al., 2004). Wnt3a CM supports self-renewal indefinitely in the absence of LIF, in contrast to CM from a non-secreting L-fibroblast cell line that has no maintenance activity (Sato et al., 2004). Under conditions where ES cells were maintained by Wnt3a CM, Myc protein levels were elevated and unphosphorylated on T58; furthermore, GSK3β kinase activity was low (Fig. 2E). By day 1 after Wnt3a CM withdrawal, we observed marked elevation of Myc T58 phosphorylation and GSK3β kinase activity (Fig. 2E). Activation of GSK3β and phosphorylation of T58 both occurred well before downregulation of Oct4, consistent with earlier observations in LIF maintained ES cells. The increase in GSK3β kinase activity is therefore consistent with it participating in T58 phosphorylation, resulting in Myc degradation following Wnt3a CM withdrawal (see Discussion). These events are indistinguishable from those that occur following LIF withdrawal (see Fig. 2A).

Sustained Myc activity maintains ES cell self-renewal in the absence of LIF

Myc levels rapidly collapse following withdrawal of LIF, indicating that this may be a requirement for ES cell differentiation. We therefore asked if sustained Myc levels

could promote self-renewal in the absence of pro-maintenance factors such as LIF. We reasoned that elevated Myc transcript levels and the absence of T58 phosphorylation could be important in establishing conditions for ES cell self-renewal. To reproduce conditions in ES cells where Myc levels are elevated, we constitutively expressed a form of Myc that evaded T58 dependent proteolysis (myc^{T58A}ER) and compared this with the effects of mycER in self-renewal assays.

Clonally selected puro^{R} D3 ES cell lines expressing either Myc or Myc^{T58A} ER fusion proteins were tested for their ability to maintain ES cells over 12-15 days. Colony morphology, SSEA1 reactivity (flow cytometry), alkaline phosphatase (AP) activity and marker transcript profiling were used as initial readouts for ES cell maintenance. In the presence of 4OHT, mycER/myc^{T58A}ER ES cells retained high SSEA1 levels (Fig. 3A), a uniform dome-shaped colony morphology (Fig. 3B) and elevated alkaline phosphatase activity (Fig. 3C) for the first 6 days (two passages). mycER colonies became more flattened

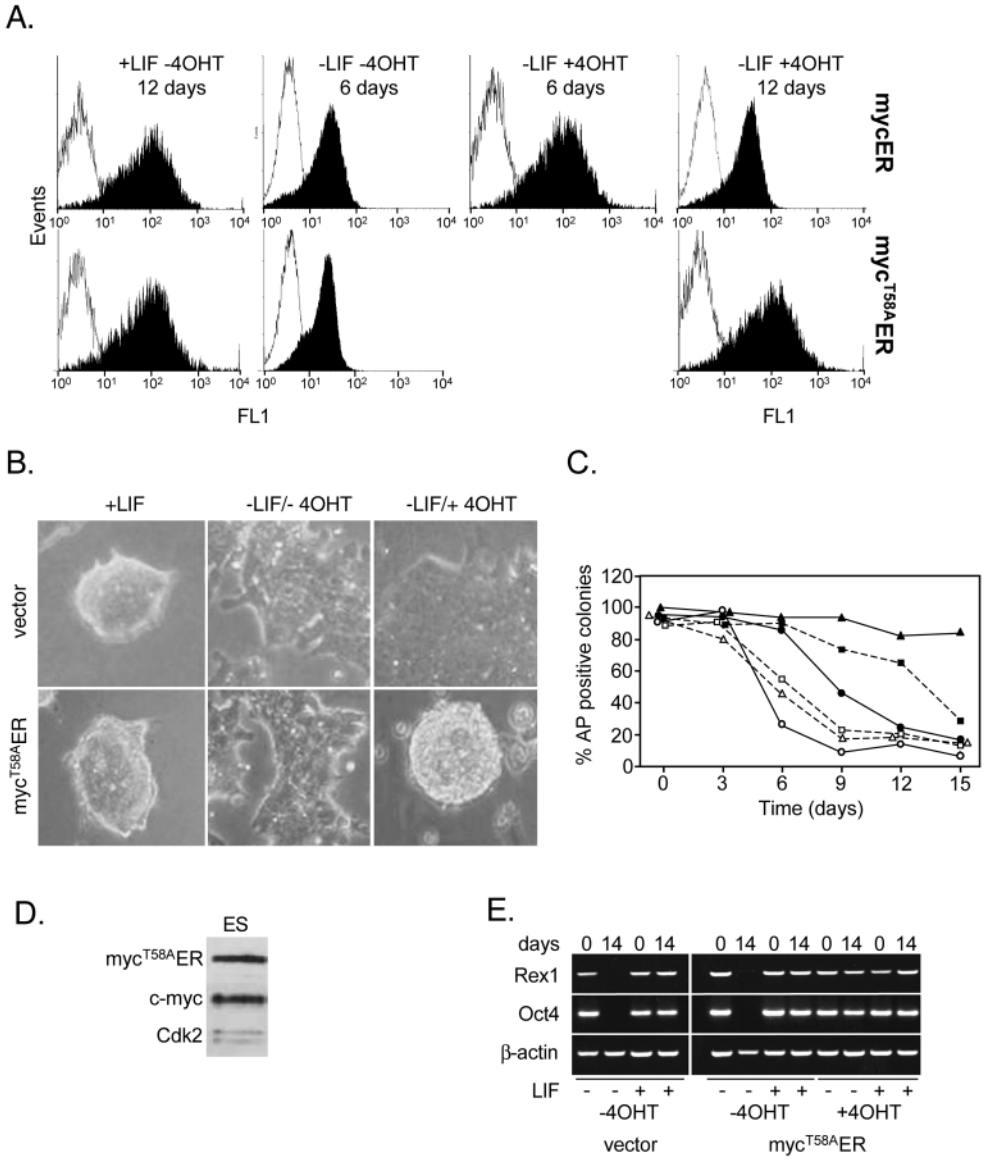

Fig. 3. Maintenance of stable Myc at physiological levels sustains self-renewal in the absence of LIF/Wnt3a CM. Myc expression was driven by the CAG promoter as a fusion with the steroid binding domain of the estrogen receptor (ER) for inducible activity and coupled to a puromycin resistance gene (puro^{R}) by an internal ribosome entry site (IRES). (A) Flow cytometry profiles of SSEA1 reactivity on the surface of mycER or myc^{T58A}ER cells grown up to 12 days in the presence of LIF (+LIF) without 4OHT (–4OHT), or without LIF (–LIF) in the presence or absence of 4OHT. (B) Colony morphology of myc^{T58A}ER transfected ES cell colonies grown in the presence or absence of 4OHT 6 days after LIF withdrawal. (C) MycER (circles, squares) and myc^{T58A}ER ES cells (triangles) were grown in varying concentrations of LIF (0-1×10^{3} units/ml), in the presence (black symbols) or absence (white symbols) of 4OHT. Colonies were assayed for AP activity every 3 days after LIF withdrawal in duplicate. MycER cells were also grown in the presence of 7.5 units/ml LIF with (closed square) or without (open square) 4OHT. (D) Immunoblot of cell lysate from the myc^{T58A}ER transfected D3 ES cell line showing levels of endogenous Myc, myc^{T58A}ER and Cdk2 after being probed simultaneously with anti-Myc and Cdk2 antibody, and then exposed to film for the same length of time. (E) RT-PCR analysis of Oct4, Rex1 and β-actin transcripts in vector alone or myc^{T58A}ER cells grown for 14 days in the presence or absence of LIF and 4OHT.

and heterogeneous with time and eventually displayed low AP and SSEA1 levels although myc^{T58A}ER cells retained these characteristics for over 12-15 days (4-5 passages; Fig. 3A-C). Including low levels of LIF (7.5 units/ml), which by themselves have only a small effect on maintaining AP levels over 15 days, appeared to further delay the loss of AP activity in mycER cells grown with 4OHT, indicating that LIF can cooperate with Myc (Fig. 3C). Expression levels of myc^{T58A}ER were comparable with that of endogenous Myc in the cell lines tested (Fig. 3D). Transcript profiling showed that myc^{T58A}ER cells retained elevated levels of ES cell mRNA transcripts Oct4 and Rex1 in a 4OHT-dependent manner throughout the experiment (Fig. 3E). Addition of 4OHT to control cells had no role in promoting self-renewal, as determined by colony morphology, transcript profiling and AP activity (Fig. 3B,E). In parallel experiments, cell lines constitutively expressing Myc^{T58A} from the CAG promoter were shown to promote self-renewal for more than 15 days (P.C. and S.D., unpublished), indicating that the ER per se is not contributing to the ability of Myc to promote self-renewal. When these experiments were repeated in R1 ES cells, we obtained similar results, indicating that Myc-dependent self-renewal is not cell line-dependent (see data in Figs 4, 5). We also confirmed that Myc-dependent self-renewal was completely independent of any STAT3 signaling by showing that myc^{T58A}ER could bypass the dominant-negative effects of STAT3^{Y705F} (see Niwa et al., 1998) (S.D., unpublished). These results indicate that the stable form of Myc normally seen in ES cells is required for maintenance of self-renewal and stem cell identity, and is likely to work downstream of LIF/STAT3.

Myc promotes self-renewal by blocking differentiation

Myc has well characterized roles in cell cycle control and apoptosis, raising the possibility that its role in promoting self-renewal/expansion of ES cells was due to an altered balance between cell death and cell division. The T58A mutation, in particular, has previously been reported to exhibit full transforming capacity but has reduced pro-apoptotic activity in comparison with normal Myc (Chang et al., 2000). To gain insight into the biological role of Myc in ES cells, we first determined proliferative rates, cell cycle kinetics and cell viability of vector alone and myc^{T58A}ER transfected ES cell lines in the presence or absence of 4OHT. Generation times, cell cycle kinetics and the extent of cell death in bulk cultures were comparable between different cell lines maintained by either LIF or 4OHT (Fig. 4A-C). The only noticeable effect of myc^{T58A} activity was a slight reduction in the percentage of G1 cells (Fig. 4B). At high 4OHT levels (>20 nM) cell death was elevated, arguing against a

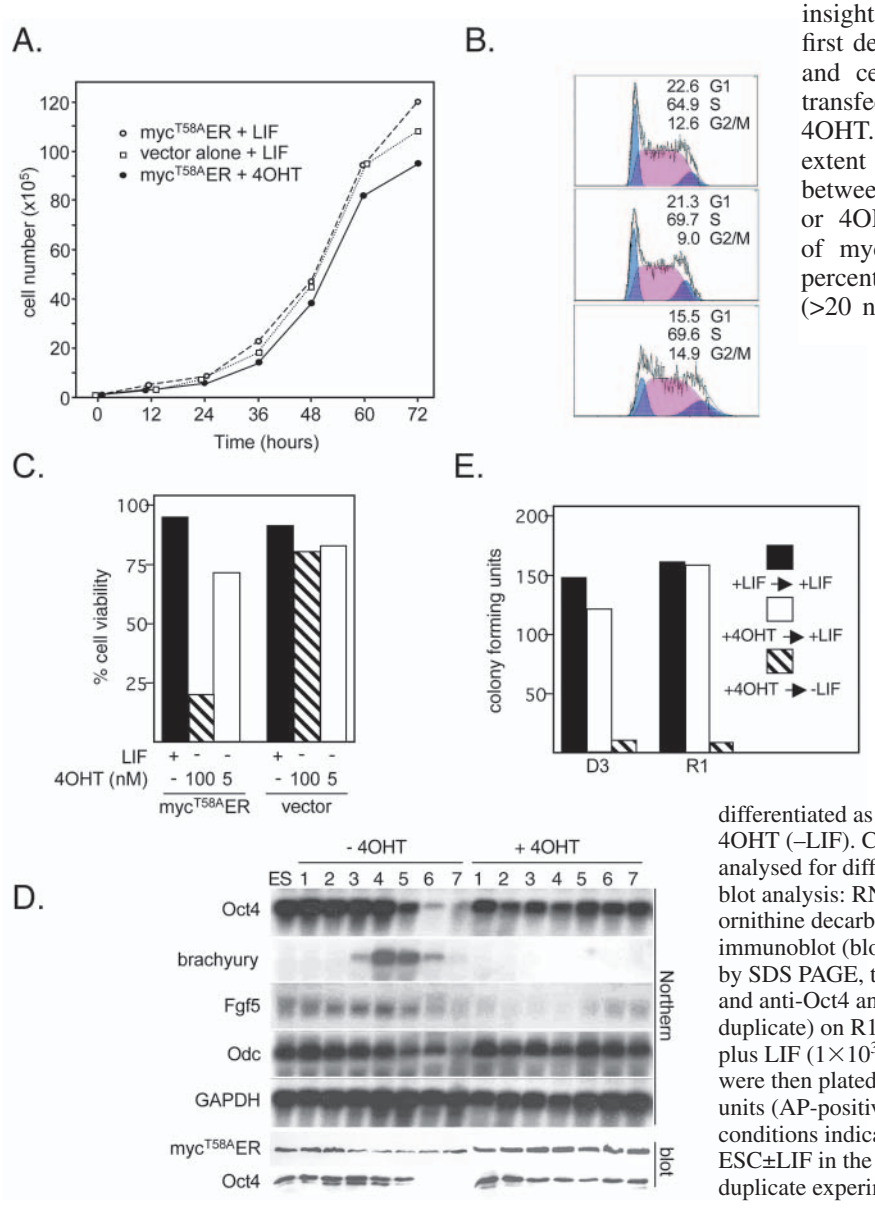

Fig. 4. Myc^{T58A}ER-dependent self-renewal is independent of cell cycle and apoptotic effects and is reversible by withdrawal of 4OHT. (A) ES cells were plated at 5×10^5/ml in duplicate wells and scored at 12-hour intervals for 72 hours. (B) Flow cytometry analysis of PI stained cells. Top panel, myc^{T58A}ER cells + LIF (1×10^3 units/ml); middle panel, vector alone + LIF (1×10^3 units/ml); bottom panel, myc^{T58A}ER + 4OHT (5 nM). The percentage of cells in different cell cycle phases was determined using MultiCycle AV software (Phoenix Flow Systems). (C) Cell viability was determined by counting 1000 cells for Trypan Blue exclusion. (D) Myc^{T58A}ER ES cells were maintained in the presence of 4OHT for 14 days (–LIF) then differentiated as embryoid bodies in either the absence or presence of 4OHT (–LIF). Cells were harvested for each of 7 days (1-7) and analysed for differentiation status as indicated. Upper panel, northern blot analysis: RNA samples probed for Oct4, brachyury, Fgf5, ornithine decarboxylase (Odc) and GAPDH. Lower panel, immunoblot (blot) analysis (blot): 40 μg total protein was resolved by SDS PAGE, transferred to a membrane then probed with anti-Myc and anti-Oct4 antibodies. (E) Colony-forming assays (performed in duplicate) on R1 and D3 ES cells passaged for 30 days in ESC media plus LIF (1×10^3 units/ml) or ESC media with 10 nM 4OHT. Cells were then plated at clonal density and after 6 days colony forming units (AP-positive colonies) were scored after growth under the conditions indicated (either maintained in ESC+LIF, or switched to ESC±LIF in the absence of 4OHT). Assays are shown as the mean of duplicate experiments.

role for reduced cell death in Myc-dependent ES cell expansion (data not shown). At the low levels of 4OHT used in these experiments (<10 nM), cell cycle kinetics and cell death were comparable with that of LIF-maintained cells. Changes in the balance between cell proliferation and cell death therefore cannot account for the ability of Myc to promote ES cell self-renewal.

To determine whether Myc-dependent self-renewal is reversible, we asked if ES cells maintained by myc^{T58A}ER for 14 days (–LIF) could differentiate following withdrawal of 4OHT. This was tested by plating cells in suspension under conditions where EBs could form for 7 days in the presence or absence of 4OHT. Under conditions where cells were grown in the presence of 4OHT, Oct4 mRNA and Oct4 protein remained elevated and differentiation markers such as Fgf5 and brachyury mRNAs remained low (Fig. 4D). After withdrawal of 4OHT, however, Oct4 mRNA and Oct4 protein levels decreased significantly, whereas Fgf5 and brachyury transcripts increased (Fig. 4D). These results indicate that myc-maintained ES cells differentiate when Myc is inactivated by removal of 4OHT.

As another test that Myc-dependent self-renewal was reversible, we performed colony forming assays on D3 and R1 ES cells grown for 30 days in the presence or absence of 4OHT (±LIF) to determine the proportion of stem cells present after inactivation of Myc. Cells were plated at clonal density under various conditions and evaluated by colony-forming assay. No major differences were observed between the number of colony-forming cells in LIF versus 4OHT maintained R1 or D3 ES cells (Fig. 4E), indicating that Myc can maintain the ES cell state at a comparable level with LIF. Removal of 4OHT (and LIF) led to a decrease in the number of colony-forming cells in bulk cultures, indicating that when Myc is inactivated, the stem cell pool declines as a result of Myc-dependent self-renewal being reversible.

The remaining scenario that could most probably account for Myc-dependent self-renewal is that it exerts its effects by imposing a blockade on differentiation. If this were the case, we would predict that ES cells would become unstable under conditions when Myc activity is reduced, leading to differentiation. To investigate this possibility we generated stable cell lines expressing a mycER fusion where the Myc open reading frame lacked most of its transactivation domain (myc$^{\Delta 40\text{-}178}$ER). This and similar dominant-negative versions of Myc have previously been shown to promote differentiation in other cell types without necessarily imposing a cell cycle arrest (Canelles et al., 1997; Schreiner et al., 2001). Although myc$^{\Delta 40\text{-}178}$ER ES cell colonies could be maintained with LIF indefinitely in the absence of 4OHT, morphologically they showed obvious signs of differentiation and loss of AP staining following the addition of 4OHT to bulk cultures (Fig. 5A,B). This was associated with a significant decrease in the ability to form AP-positive colonies when cells were plated at clonal density (Fig. 5C). These data suggest that the proportion of self-renewing stem cells had significantly declined as a consequence of decreased Myc activity, indicating that differentiation had occurred. These findings were confirmed by showing that expression of specific transcripts associated with pluripotency, such as Nanog and Oct4, declined following addition of 4OHT and that mesoderm (brachyury), GATA4 (extra-embryonic endoderm) transcripts were expressed at higher levels (Fig. 5D). A predominance of extra-embryonic endoderm and mesoderm is typically produced following LIF withdrawal from adherent ES cell cultures (Niwa et al., 2000) and although mRNA markers for these lineages were detected, we did not perform analysis at the single cell level to determine the percentage of different cell types being generated. We

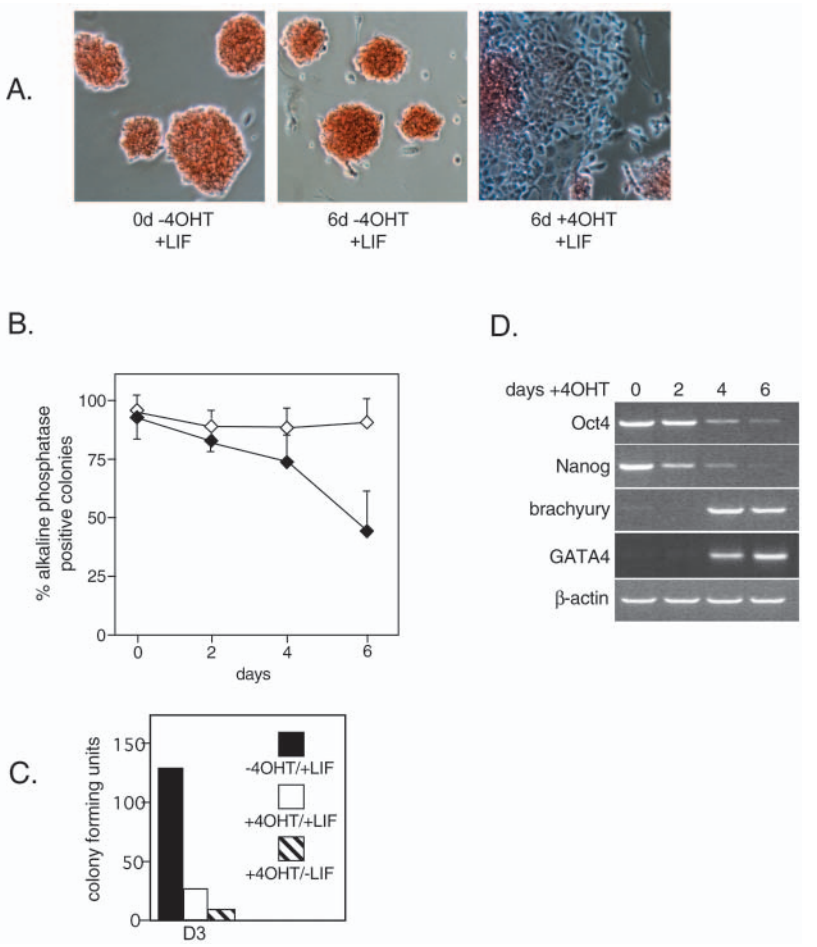

Fig. 5. Myc performs a role in self-renewal by blocking differentiation. (A) Myc$^{\Delta 40\text{-}178}$ER D3 cells were grown in the presence of LIF and in the absence or presence of 4OHT (25 nM) for up to 6 days. Colonies were then stained for AP activity and photographed at 20× magnification. (B) At corresponding sampling times (see A) the percentage of alkaline phosphatase positive colonies was determined. Data are the average of assays performed in triplicate and expressed as standard error of the mean. (C) The proportion of stem cells decreases following inactivation of Myc. Myc$^{\Delta 40\text{-}178}$ER D3 ES cells were grown for 6 days under the conditions indicated. Five-hundred cells were then plated at clonal density in ESC+LIF (1×10^3 units/ml) and colony forming units (AP-positive colony number) scored after a further 4 days growth (–4OHT). Data are shown of an experiment performed in duplicate. (D) mRNA levels for Oct4, Nanog, brachyury, GATA4 and β-actin were evaluated by RT-PCR analysis in D3 myc$^{\Delta 40\text{-}178}$ER cells before and after addition of 4OHT.

interpret our data to indicate that abrogation of Myc activity is incompatible with maintenance of ES cell self-renewal. Together with our previous findings, we conclude that Myc is necessary and sufficient for ES cell self-renewal.

Myc maintained ES cells retain wide-range differentiation potential

We next evaluated the ability of myc^{T58A}ER maintained (+4OHT) EGFP R1 cells (Hadjantonakis et al., 1998) to differentiate in an in vivo setting by asking if they could contribute to the three embryonic germ layers following injection into blastocyst stage embryos. Cells were first maintained in 4OHT for 30 days and grown as EBs in the absence of LIF and 4OHT to confirm they differentiate in vitro by monitoring the downregulation of Oct4 protein (Fig. 6A). In parallel to this, cells were directly injected into C57BL/6 blastocyst stage embryos. myc^{T58A}ER cells maintained for 30 days in 4OHT were confirmed to be AP positive and, when plated onto gelatin coated plastic in the absence of 4OHT, did not form domed-shaped colonies but formed non-uniform, flattened monolayers indicative of differentiation (Fig. 6B). Following injection of EGFP-marked R1 ES cells into C57BL/6 blastocysts, embryos were analyzed at 12.5 dpc for their integration into the three germ layers. The extensive integration of myc^{T58A}ER EGFP-marked cells throughout the embryo indicates that they contributed to all three embryonic germ layers and hence, retained pluripotency (Fig. 6C-F). A mock-injected embryo is shown to demonstrate negligible autofluorescence (Fig. 6C,D). Injected embryos that developed to term generated live chimeric animals as judged by chimeric coat color (Fig. 6F). The relative distribution and degree of chimerism of myc^{T58A}ER cells was indistinguishable from non-transfected EGFP ES cell lines (Fig. 6F). From a total of 18 injected embryos, more than 90% were judged to be chimeric by EGFP incorporation. At least ten chimeric animals were generated for each cell line, each displaying chimeric coat color. Similar results were obtained with myc^{T58A}ER maintained D3 ES cell lines (P.C. and S.D., unpublished). We cannot rule out the possibility that Myc-maintained ES cells may be more predisposed to differentiate into certain lineages over others. We have shown that they can differentiate into cell types representative of the three germ layers and retain wide-range differentiation potential, even though germline transmission was not demonstrated. These analyses confirm, however, that Myc-dependent self-renewal is reversible and identify Myc as a central maintenance factor of ES cell pluripotency.

Discussion

Potential mechanisms of myc-dependent self-renewal

Embryonic stem (ES) cells have the capacity for unlimited proliferation and differentiation into a wide range of cell types. Although it is known that all of the essential functions of LIF dependent self-renewal depend on STAT3, the downstream effectors of this pathway have not been defined previously. Our data clearly establishes a role for Myc in self-renewal and maintenance of ES cell pluripotency by functioning as the key target of LIF-STAT3 signaling. Although Myc has been

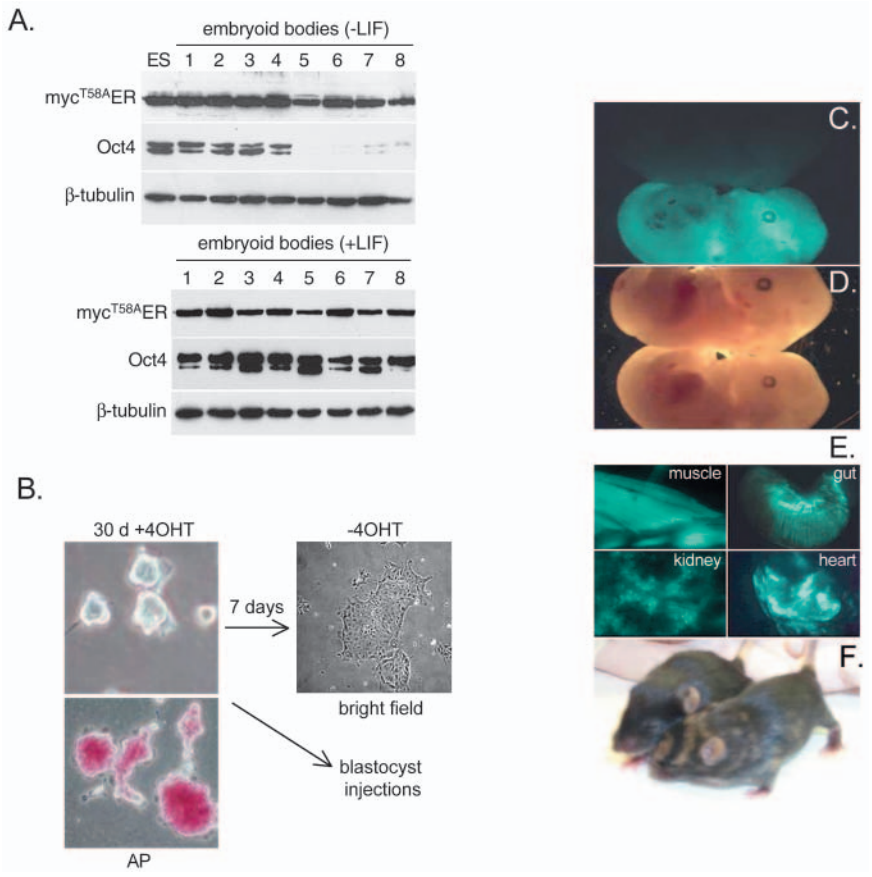

Fig. 6. Myc-maintained ES cells retain pluripotency. (A) Myc^{T58A}ER R1 EGFP ES cells were maintained in the presence of 4OHT for 30 days, in the absence of LIF/Wnt3a CM and then grown as EB suspensions for up to 8 days by withdrawing 4OHT in the absence (top panel) or presence (bottom panel) of LIF. Cell lysates were subject to immunoblot analysis, probing with anti Myc, Oct4 and β-tubulin antibodies. (B) Myc^{T58A}ER ES cells grown in the presence of 4OHT for 30 days (as in A) were stained for AP activity or grown for an additional 7 days in the absence of 4OHT. (C) GFP marked R1 myc^{T58A}ER ES cells grown for 30 days in the presence of 4OHT (–LIF; as in A) then used for injection into blastocyst stage C57BL/6 embryos that were transferred into females and allowed to develop to 12.5 dpc, at which time they were analysed. Chimeric (bottom) and non-chimeric (top) embryos where EGFP R1 myc^{T58A}ER maintained ES cells had widely integrated into the three germ layers. (D) Dark field view of the same embryos. (E) Tissues were dissected from EGFP-positive embryos and analyzed at higher magnification. (F) The ability of myc^{T58A}ER ES cells to generate liveborn chimeras was confirmed by analysis of coat color. Host C57BL/6 embryos give rise to black coat color, whereas sv129 (R1 ES line) contribute to a sandy-colored coat. Chimeras shown were generated by contribution of LIF maintained (left) and 4OHT maintained (right) myc^{T58A}ER cells.

reported to play a role in influencing stem cell behavior in various contexts (Waikel et al., 2001; Frye et al., 2003; Satoh et al., 2004), we provide the first evidence that Myc plays a central role in maintenance of ES cell identity. Our observations that Myc can maintain ES cell self-renewal is paralleled by its well documented role in cell immortalization. It is notable then that orchestrators of tumor cell immortalization may overlap with those which regulate stem cell self-renewal and suggests that similar mechanisms may operate. One Myc target that is involved in immortalization and self-renewal is TERT, the regulatory subunit of telomerase (Wang et al., 1998). To our knowledge, work in this report establishes Myc as the first regulator of both ES cell self-renewal and cell immortalization.

A problem in deciphering the true function of Myc has been its promiscuous role in a wide variety of transcriptionally regulated pathways that generate a diversity of biological outcomes (see Patel et al., 2004). The identification of Myc target genes has been particularly problematic, with over 20 transcription profiling papers failing to reach a clear consensus with regards to bone fide genomic targets (Oster et al., 2002). The multiplicity of such data underscores the pleiotropic functions of this transcription factor, indicating that its biological roles are highly cell-type specific and subject to complex regulation by extracellular signals and by its binding to cellular co-factors. Analysis of binding sites in human and *Drosophila* cells in vivo shows that Myc is associated with an extraordinarily large number of genomic sites (Fernandez et al., 2003; Orian et al., 2003), indicating that it can participate in the regulation of complex genetic pathways. Although Myc may bind overlapping sets of genes under various conditions in different cell types (Fernandez et al., 2003), transcriptional responses to Myc binding differs markedly in a context and cell type-dependent manner (Ellwood-Yen et al., 2003). In the case of ES cells, Myc family members may bind and regulate a cluster of genes required for stem cell maintenance that would be only partially overlapping with those regulated by Myc in other biological contexts. Genes identified previously as being specifically associated with the pluripotent state (Calaveri and Scholer, 2003) may in fact be part of the Myc regulatory hierarchy in ES cells.

Although stem cell markers such as Oct4 and Nanog persist for several days following LIF withdrawal, ES cells are committed to differentiate within 24 to 36 hours (Boeuf et al., 2001). This implies that the commitment 'decision' occurs long before Oct4 or Nanog levels are extinguished. The ability of Myc to maintain self-renewal combined with its rapid downregulation within the first 36 hours following LIF withdrawal, suggests it to be a key determinant of commitment and could provide the molecular basis underpinning the commitment phase. Hence, the inactivation of Myc transcriptional regulators and the activation of pathways required for Myc degradation could define a 'point of no return' where cells become committed to differentiate. We predict that if mycER was reactivated within this 'window' following LIF or 4OHT withdrawal, ES cells would retain their stem cell identity but this would not be possible if restoration of Myc activity was further delayed.

A direct role for Myc in development of pluripotent cells in the preimplantation embryo has not been previously described nor has it been implicated through gene knockout studies in the mouse (Davis et al., 1993; Stanton et al., 1992), even though Myc family members are expressed at elevated levels during this phase of development (Downs et al., 1989). A likely explanation for this is that functional redundancy among the Myc family masks the function of individual members during early development in loss-of-function experiments (Malynn et al., 2000). This is consistent with our observations that Nmyc1 can substitute for Myc in promoting self-renewal of ES cells and that Nmyc1 is regulated in parallel to Myc at the transcript and protein level (S.D., unpublished). It is not known, however, if Myc and Nmyc1 are subject to the same regulatory pathways in pluripotent cells.

Two lines of evidence support a case for involvement of Myc activity in the development of pluripotent cells in the embryo. First, enforced expression of an oncogenic form of L-myc (Rlf) blocks normal development of the embryonic epiblast (MacLean-Hunter et al., 1994). Second, the obligatory binding partner of Myc, Max, is essential for early embryonic growth and development (Shen-Li et al., 2000). The essential role of Max in the early embryo therefore implicates an important role for Myc family members in development of the embryonic epiblast that would be compatible with it having a role in maintenance and regulation of the pluripotent state. Pools of maternally derived Myc family members persisting during early embryonic development could also potentially provide additional levels of redundancy to ensure the correct progression of pluripotent cells, reinforcing the robust nature of embryonic development.

Multiple levels of control regulate the Myc dependent ES cell self-renewal pathway

Under conditions where ES cells are maintained by Wnt3a CM, Myc levels are elevated, T58 is unphosphorylated and GSK3β activity is suppressed. These trends are rapidly reversed following Wnt3a CM withdrawal, similar to events following LIF withdrawal. This raises the possibility that LIF and Wnt signaling pathways converge on Myc as a common target to promote self-renewal. Self-renewal of ES cells is dependent on the regulation of Myc at two levels. First, through transcriptional activation of the Myc gene; and, second, by establishment of conditions where Myc stability is enhanced through inhibition of T58 phosphorylation (Fig. 7). Although the LIF-STAT3 pathway is clearly critical for maintenance of Myc transcription in ES cells, it probably functions in collaboration with additional signaling pathways, such as those generated by serum-derived factors. The dual mode of Myc regulation is similar to that recently reported for regulation of Nmyc1 in cerebellar precursor cells, where it plays a role in promoting cell division (Kenney et al., 2004). In this scenario, transcription is activated by sonic hedgehog through an undefined mechanism and by increased protein stability achieved through inhibition of GSK3 by PI3 kinase.

When maintained by LIF, transcriptional control of Myc in ES cells is under the direct control of STAT3. Wnt is likely to achieve the same outcome by a β-catenin/TCF-LEF-dependent mechanism (He et al., 1998), which raises the possibility that STAT3 and β-catenin/TCF-LEF perform equivalent roles in promoting self-renewal. The second level of control depends on the suppression of GSK3β activity in ES cells through an undefined mechanism. Although our studies explain how LIF and Wnt3a CM can contribute to ES self-renewal through transcriptional mechanisms, they do not resolve the issue of how GSK3β is suppressed in the presence of LIF/Wnt but

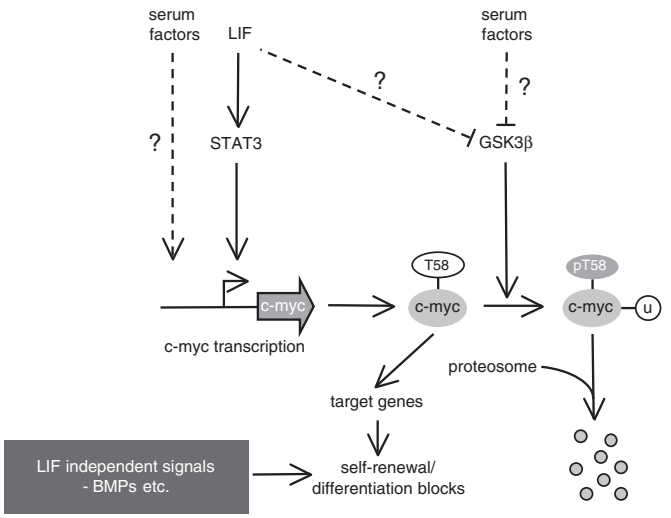

Fig. 7. The relationship between LIF-STAT3, GSK3β and Myc in maintenance of ES cell self-renewal. Following LIF withdrawal, Myc transcriptional activity collapses, GSK3β is activated and Myc becomes phosphorylated at T58, triggering its ubiquitin (u)-mediated, proteosome-dependent degradation. '?' indicates the possible role of pathways involved in (1) suppression of GSK3β activity or (2) pathways of transcriptional control that collaborate with LIF to control Myc transcription. The latter could involve signals generated by serum-derived growth factors. The involvement of LIF/STAT3-Myc independent pathways are also indicated.

rapidly activated following the removal of pro-maintenance signals. Both gp130- and Wnt-dependent signaling pathways, however, have been previously implicated in negative regulation of GSK3, providing a plausible explanation to account for how Myc stability is maintained in the presence of LIF- and Wnt-dependent signals (Takahashi-Tezuka et al., 1998). More recently, LIF signaling has been shown to activate PI3K, resulting in the suppression of GSK3β activity in murine ES cells (Paling et al., 2005). This would explain our results showing that GSK3β is rapidly activated following LIF withdrawal and how Myc is phosphorylated on T58 (see Fig. 7). Although our model proposes that at least two pathways must operate to establish conditions where Myc can promote self-renewal, there are additional pathways that function in parallel to the LIF/STAT3/Myc to sustain pluripotency (see Introduction, Fig. 7). How all of these signals are integrated to generate a defined biological outcome – self-renewal – is not understood.

Degradation of Myc requires its phosphorylation by GSK3β on T58,, leading to its ubiquitin-dependent degradation (Sears et al., 2000; Gregory et al., 2003). The requirement for a stable form of Myc in ES cell self-renewal is made more intriguing as GSK3β inhibition was recently shown to promote self-renewal of mouse and human ES cells in the absence of LIF or Wnt (Sato et al., 2004). These data suggested to us that suppression of GSK3β and enhanced Myc stability could be linked. A role for GSK3β in promoting differentiation through T58-dependent degradation is consistent with the kinetics of GSK3β activation and Myc T58 phosphorylation following LIF/Wnt3a CM withdrawal. These observations point towards a scenario where maintenance of ES cells requires elevated Myc levels, achieved in part through the suppression of GSK3β activity. Activation of GSK3β following LIF/Wnt3a CM withdrawal can then account for decreased Myc stability and loss of a stable self-renewal pathway. The stability of Myc in ES cells in unprecedented and is comparable with the stability of oncogenic forms of the protein associated with Burkitt's lymphoma. Our findings also provide a potential mechanism for how the GSK3 inhibitor 6-bromoindirubin-3′-oxime (BIO) promotes self-renewal of ES cells (Sato et al., 2004). We predict that suppression of GSK3 activity by BIO, in the absence of LIF/Wnt signaling, would establish conditions where Myc was unphosphorylated on T58, leading to elevated Myc levels and enhanced stem cell stability. This mechanism may also be applicable to human ES cells where BIO has a pro-maintenance function (Sato et al., 2004).

Our findings establish a mechanism for how ES cells retain the ability to proliferate indefinitely while retaining their pluripotentiality. The identification of Myc as a key regulator of ES cell self-renewal raises two important questions. First, does Myc have a role in maintenance of human ES cells? Second, does Myc have a role in self-renewal in other stem cells that use Wnt-dependent mechanisms? These questions are currently being addressed.

This work was supported by the Georgia Research Alliance and the Georgia Cancer Coalition. We thank Anna Kenney, Hitoshi Niwa and Shinya Yamanaka for critical review of the manuscript and valuable suggestions; and David Martin for performing blastocyst injections.

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