Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency

Elena Carbognin1,†, Riccardo M Betto1,†, Maria E Soriano2, Austin G Smith3,4,* & Graziano Martello1,**

Abstract

Transcription factor Stat3 directs self-renewal of pluripotent mouse embryonic stem (ES) cells downstream of the cytokine leukemia inhibitory factor (LIF). Stat3 upregulates pivotal transcription factors in the ES cell gene regulatory network to sustain naive identity. Stat3 also contributes to the rapid proliferation of ES cells. Here, we show that Stat3 increases the expression of mitochondrial-encoded transcripts and enhances oxidative metabolism. Chromatin immunoprecipitation reveals that Stat3 binds to the mitochondrial genome, consistent with direct transcriptional regulation. An engineered form of Stat3 that localizes predominantly to mitochondria is sufficient to support enhanced proliferation of ES cells, but not to maintain their undifferentiated phenotype. Furthermore, during reprogramming from primed to naive states of pluripotency, Stat3 similarly upregulates mitochondrial transcripts and facilitates metabolic resetting. These findings suggest that the potent stimulation of naive pluripotency by LIF/Stat3 is attributable to parallel and synergistic induction of both mitochondrial respiration and nuclear transcription factors.

Keywords LIF, metabolism; mitochondrial respiration; pluripotency; Stat3

Introduction

Mouse embryonic stem (ES) cells (Evans & Kaufman, 1981; Martin, 1981) have the capacity to give rise to all differentiated cells of the body and the germ line (Bradley et al., 1984), a feature termed pluripotency (Bradley et al., 1984; Martello & Smith, 2014). ES cells are derived from the naive pluripotent epiblast of mouse blastocysts (Brook & Gardner, 1997; Boroviak et al., 2014). The cytokine leukemia inhibitory factor (LIF) is pivotal for establishing and maintaining ES cells in culture (Smith et al., 1988; Williams et al., 1988; Nichols et al., 1994). LIF signals via the LIF-R/gp130 complex, which activates Janus-associated kinases (JAKs) (Burdon et al., 2002). In turn, JAKs phosphorylate and activate the transcription factor Stat3, which maintains naive pluripotency through its direct targets Tclp21, Klf4, and Gbx2 (Niwa et al., 1998, 2009; Bourillot et al., 2009; Martello et al., 2013; Tai & Ying, 2013), key members of the ES cell core gene regulatory network (Dunn et al., 2014).

Blockade of GSK3 and MEK kinases permits ES cell self-renewal in the absence of LIF (Ying et al., 2008; Martello et al., 2013). Importantly, however, self-renewal efficiency is significantly increased when LIF is added (Wray et al., 2010; Dunn et al., 2014). LIF/Stat3 signaling is also critical during cellular reprogramming to facilitate the attainment of naive pluripotency (Takahashi & Yamanaka, 2006; Yang et al., 2010; van Oosten et al., 2012; Martello et al., 2013; Stuart et al., 2014).

Naïve pluripotent cells are metabolically flexible as they utilize both glycolysis and mitochondrial respiration (Zhou et al., 2012; Teslaa & Teitell, 2015). However, this is not a feature of all pluripotent cells. EpiSCs derived from the primed epiblast of post-implantation embryos (Brons et al., 2007; Tesar et al., 2007; Nichols & Smith, 2009) are mainly glycolytic with inert mitochondria (Zhou et al., 2012). The switch from aerobic to anaerobic metabolism presumably reflects the altered environment of the embryo upon implantation, but is evidently intrinsically programmed.

Here, we investigate the impact of LIF/Stat3 on mitochondrial activity during mouse ES cell propagation and reprogramming from primed to naive pluripotency.

Results

The LIF/Stat3 axis promotes ES cell proliferation and mitochondrial transcription

Embryonic stem cells can be derived and expanded under feeder-free conditions in the presence of two inhibitors (2i) with or without
LIF (Ying et al., 2008; Wray et al., 2010). ES cells expanded in 2i retain the ability to form chimeric animals and be transmitted through the germline, indicating that LIF signaling can be dispensable for the maintenance of pluripotency in vitro, although LIF dependency varies with genetic background. Consistent with this, Stat3 null ES cells have been previously derived and characterized in 2i and showed no overt defects in early lineage differentiation or self-renewal capacity (Ying et al., 2008; Wray et al., 2011; Martello et al., 2013). Nonetheless, addition of LIF to 2i (2i + LIF) is beneficial to the culture of wild-type ES cells, resulting in increased clonogenicity (Wray et al., 2010; Dunn et al., 2014) and more robust and rapid expansion (Fig 1A).

We investigated whether the effect of LIF on population doubling was due to an increase in cell survival or in proliferation rate. We found that the percentage of viable cells was not affected (Appendix Fig S1A), but that LIF caused a reduction in the fraction of cells in G1 phase, with a concomitant increase in actively dividing cells (Fig 1B).

LIF is known to activate three signaling pathways, Stat3, PI3K, and Erk, each of which could mediate an effect on proliferation (Burdon et al., 1999). Presence of the Mek inhibitor in 2i rules out a contribution of the Erk cascade. We took advantage of Stat3 null cells and found that their proliferation rate is not increased by LIF and is comparable to that of wild-type cells cultured without LIF (Fig 1A). We conclude that Stat3 is required for the proliferative response to LIF.

We analyzed transcriptome data from mES cells cultured in 2i and stimulated with LIF for 1 h (Martello et al., 2013) to identify transcriptional targets that might be related to the effects on proliferation. We found that several mitochondrial transcripts were elevated in response to LIF (Fig 1C). In particular, mRNAs coding for subunits of the complexes of the mitochondrial respiratory chain were upregulated around twofold by LIF treatment (Fig 1D). This effect was not observed in Stat3 null cells. These results were validated by quantitative real-time PCR (RT-qPCR) on cells either acutely stimulated with LIF or kept in 2i + LIF conditions for 2 passages, the latter result indicating that the response is stable over time (Fig 1E, top).

LIF/Stat3 could enhance mitochondrial transcription indirectly, via induction of known mitochondrial master transcriptional regulators, such as PGC-1 or TFAM. Inspection of the RNA-seq data from LIF stimulation showed no induction of either of these regulators (Appendix Fig S1C).

To explore whether the effect of LIF/Stat3 on mitochondrial transcription may be direct, we designed a reporter assay. A single regulatory region, the D-loop, directs transcription of the mitochondrial genome. We generated a reporter construct containing the mouse D-loop followed by a minimal promoter and the firefly luciferase ORF (D-loop-Lux, Fig 2A) and introduced this into both ES cells and EpiSCs. In either case, cotransfection with Stat3 increased reporter activity (Fig 2B and C). EpiSCs showed more pronounced reporter activation, probably due to lower levels of endogenous Stat3 pathway.

To examine further whether Stat3 could directly regulate mitochondrial transcription, we inspected available chromatin immunoprecipitation followed by sequencing (ChIP-seq) data (Sánchez Castillo et al., 2015). We observed a significant enrichment of Stat3 over the D-loop region of the mitochondrial genome (Fig 2D). We performed ChIP-qPCR and confirmed binding of Stat3 at the D-loop in mES cells (Fig 2E).

Mitochondrial genomes exist as clusters associated with specific proteins, termed nucleoids, that lie within the mitochondrial matrix. Atad3 is a protein required for correct nucleoid assembly which interacts with the D-loop region (He et al., 2007). We first confirmed that Atad3 and mtDNA colocalized in mES cells (Appendix Fig S2A). We used the proximity ligation assay (PLA) to test for colocalization of endogenous Stat3 and Atad3. The results in Fig 2F indicate that the two proteins are closely associated within mitochondria in ES cells.

Collectively, these findings suggest that Stat3 directly induces transcription of the mitochondrial genome, but do not rule out other potential effects of Stat3 on the stability or turnover of mitochondrial transcripts.

**Mitochondrial respiration is increased in the presence of LIF**

We investigated whether alterations in the level of mitochondrial transcription are accompanied by altered respiratory activity. Stat3 was previously shown to be a positive regulator of mitochondrial respiration in terminally differentiated cells (Wegrzyn et al., 2009; Zouein et al., 2014). We measured the oxygen consumption rate (OCR) in wild-type and Stat3 null cells cultured in 2i + LIF by extracellular flux analysis (Seahorse assay). In the absence of Stat3, we found a reduction both in the basal levels of OCR and after treatment with the uncoupler FCCP, which provides a measure of the maximal respiratory rate (Figs 3A and Appendix Fig S3A). These
Stat3 activates mitochondria in pluripotent cells

Elena Carbognin et al

The EMBO Journal

Figure 1.
Figure 2.
results prompted us to assess whether the positive effect of Stat3 on mitochondrial respiration requires active LIF signaling or may be a constitutive function of Stat3 independent of the signaling context. We measured OCR in cells cultured for multiple passages in either 2i or 2i + LIF and observed an increase in both basal and maximal respiration in the presence of LIF (Fig 3B and C). Under the same conditions, we measured the extracellular acidification rate (ECAR), which provides an indirect measure of the glycolytic flux, and found that LIF has no consistent effect on ECAR (Appendix Fig S3B and C).

Increased respiration could be due to enhanced mitochondrial biogenesis. However, protein levels of two components of the import machinery (TOM20 and TIMM23), whose expression correlates with mitochondrial biomass, were not increased in the presence of LIF (Fig 3D), suggesting that LIF does not have a substantial influence on mitochondrial biogenesis. We also measured the number of copies of the mitochondrial genome relative to the nuclear genome by PCR in 2i or 2i + LIF and could not detect any significant difference (Fig 3E). A constant number of genomes are consistent with the elevated mitochondrial transcript levels arising from a specific increase in transcription. We focussed our attention on Complex I, which is the main entry point to the respiratory chain, because several of its subunits are transcriptionally regulated by LIF/Stat3 (Fig 1C and E). We performed blue native gel electrophoresis (BNGE) to isolate intact complexes and by Western blot observed a reduction in the levels of Complex I in the absence of LIF and still lower levels in Stat3 null cells (Fig 3F). BNGE also allows the detection of high molecular weight clusters containing several complexes, called supercomplexes (Schägger, 1995). We observed that supercomplexes are present in ES cells and that their levels are reduced in the absence of either LIF or Stat3 (RCS on Fig 3F and G). These results suggest that LIF/Stat3 increases the levels of complexes of the respiratory chain, which in turn results in enhanced assembly of supercomplexes and elevated mitochondrial respiration.

Mitochondrial respiration determines optimal proliferation

LIF/Stat3 promotes the proliferation of ES cells as well as mitochondrial respiration. We asked whether the two effects are causally linked. To this end, we first applied rotenone, an inhibitor of Complex I. We titrated rotenone and found that concentrations ranging from 50 to 100 nM were able to reduce cell proliferation (Appendix Fig S4A), also reducing OCR by ~70% without affecting ES cell viability (Appendix Fig S4B and Fig 4A). We then tested the effect of rotenone on proliferation upon perturbation of the LIF/Stat3 axis (Fig 4B). LIF increased the number of wild-type cells (Fig 4B, compare 1st and 2nd bar) and rotenone abrogated this effect (compare the 2nd bar to the 3rd and 4th bars). As expected, Stat3 null cells did not respond to LIF (5th vs. 6th bar), but they also appeared more affected by rotenone (compare 6th to the 7th and 8th bars), a result in line with their reduced basal respiratory capacity (Fig 3A). Similar results were obtained with wild-type ES cells cultured in LIF + serum (Appendix Fig S4C), suggesting that the effect described is not related to the 2i culture conditions.

As an independent test, we depleted Ndufs3, a Complex I subunit that has been shown to be required for Complex I assembly and activity (Lapuente-Brun et al, 2013), using shRNA. Ndufs3 knockdown resulted in reduced OCR levels and proliferation in response to LIF (Fig 4C–E and Appendix Fig S4D), consistent with the rotenone results.

To further confirm that the effects of rotenone are due to inhibition of the respiratory chain, we used antimycin A, an inhibitor of Complex III. Titrated doses of antimycin A were sufficient to reduce OCR without effect on cell survival (Appendix Fig S4E–G) and also potently reduced cell proliferation (Fig 4F). Similar results were obtained with myxothiazol, a second Complex III inhibitor (Appendix Fig S5A–C).

Inhibition of the respiratory chain could affect the production of reactive oxygen species (ROS), which in turn could either be cytotoxic or act as signaling molecules. We assayed the production of ROS after treatment with rotenone, antimycin A, and myxothiazol and did not detect any increase (Appendix Fig S6A–D) at the concentrations that affected ES cell proliferation. Therefore, ROS does not seem to play a role in this context.

We then tested the effects of long-term treatment with rotenone. We observed a dose-dependent reduction in the cumulative number of cells over multiple passages (Fig 4G), but without overt effects on cell survival (Appendix Fig S6E). Crucially, ES cells remained morphologically undifferentiated and maintained full expression of pluripotency factors (Fig 4H and I). Moreover, known direct transcriptional targets of LIF/Stat3 were not affected, suggesting that
rotenone does not affect LIF signaling to the nucleus in ES cells (Fig 4H, right bars).

These results indicate that mitochondrial respiration is instrumental for maximal proliferation of ES cells and furthermore suggest that LIF effects on proliferation and pluripotent cell identity may be uncoupled.

**Mitochondrial localization of Stat3 is crucial for LIF effects on proliferation**

The effects of LIF signaling on ES cell proliferation and mitochondrial activity are strictly dependent on the presence of Stat3 (Figs 1A and B, and 3A). Thus, Stat3 null cells represent a valuable tool. We
Stat3 activates mitochondria in pluripotent cells

Elena Carbognin et al

The EMBO Journal

Published online: February 22, 2016

first transfected Stat3 null cells with a full-length mStat3 ORF, randomly picked several clones, and selected two clones expressing Stat3 protein at ~twofold over endogenous wild-type levels (Fig 5A and B). Both clones reacquired the ability to respond to LIF measured by activation of the direct target Socs3 (Fig 5C) and in terms of cell proliferation (Fig 5D). When challenged with rotenone, the rescue clones proliferated more than null cells (Appendix Fig S7A). We conclude that the proliferative defects observed in Stat3 null cells are reversible and specifically due to the lack of Stat3.

We tested whether in ES cells the effects of LIF on proliferation and respiration are due to Stat3 localization to the mitochondria, or are mediated by nuclear targets of Stat3. To do so, we transfected Stat3 null cells with a construct expressing the Stat3 cDNA fused to a mitochondrial localization signal (MLS-Stat3) as previously described (Wegrzyn et al., 2009). We generated clones expressing MLS-Stat3 at similar levels to endogenous Stat3 in wild-type cells (Fig 5E, total fractions). We prepared the mitochondrial fraction from ES cells. Representation of the nuclear protein TRIM33 was reduced by >90% (Fig 5E) compared to total cell extracts, while the mitochondrial protein TOM20 was readily detectable, indicating successful isolation of mitochondria. Endogenous Stat3 protein was detected in the mitochondrial fraction of wild-type cells and MLS-Stat3 was clearly enriched in the mitochondria of transfected cells.

Immunofluorescence staining also indicated that MLS-Stat3 was present in mitochondria (Fig 5F). Conversely, double immunostaining with TOM20, a protein present on the outer mitochondrial membrane, shows adjacent but non-overlapping localization (Appendix Fig S7C), suggesting that MLS-Stat3 is located within the mitochondrial matrix.

We characterized the transcriptional response in cells expressing MLS-Stat3 and found that they did not activate the nuclear target Socs3 in response to LIF (Fig 5G, blue bars). In contrast, mitochondrial targets were activated at levels comparable to, or higher than, control cells (Fig 5G, yellow bars). By ChIP-PCR, we found a significant enrichment of MLS-Stat3 on the D-loop region of the mitochondrial genome (Fig 5H). A direct interaction between MLS-Stat3 and nucleoid structures was also evidenced by PLA (Fig 5I and Appendix Fig S7D).

Finally, we again measured the expression of master regulators of mitochondria transcription, such as TFAM, and found no significant changes (Appendix Fig S7E). Collectively, these data are consistent with direct regulation of expression of mitochondrial genes.

We examined the functional impact of MLS-Stat3 on proliferation of Stat3 null cells. All three MLS-Stat3 clones expanded more rapidly than the null cells, and two of the clones showed a similar increase in cell numbers to wild-type cells (Fig 5J). MLS-Stat3 clones also proliferated more in the presence of rotenone (Fig 5K). They showed typical compact morphology of undifferentiated ES cells and colony sizes appeared on average larger than null cells (Fig 5L). These experiments were performed in the presence of LIF. In 2i conditions that are sufficient for wild-type ES cell self-renewal (Wray et al., 2010; Dunn et al., 2014) (Appendix Fig S9A). Both null and MLS-Stat3 clones exhibited a similar expansion rate to Stat3 null cells (Appendix Fig S8A), indicating that the effect of MLS-Stat3 on ES cell proliferation requires LIF stimulation.

A minor fraction of MLS-Stat3 becomes phosphorylated on Tyr705, the JAK target site (Appendix Fig S7B), but whether this is in the mitochondria is uncertain and the mechanism for such an effect is unknown. We also noted that LIF increased total Stat3 protein levels (Appendix Fig S8B). While Stat3 is known to autoregulate its own transcription, this will not apply to the MLS-Stat3 transgene. This observation therefore suggests that another mode of LIF signaling, potentially through PI3K, may increase translation of MLS-Stat3 or stabilize the protein.

We examined whether MLS-Stat3 is able to mediate the effects of LIF on inhibition of ES cell differentiation, which is considered to be dependent on nuclear transcriptional targets (Niwa et al., 2009; Martello et al., 2013). Stat3 null cells and MLS-Stat3 clones were transferred to culture in LIF and Mek inhibitor (LIF + PD), conditions that are sufficient for wild-type ES cell self-renewal (Wray et al., 2010; Dunn et al., 2014) (Appendix Fig S9A). Both null and MLS-Stat3 cultures underwent differentiation and cell death and collapsed completely within three passages. In contrast, a Stat3 null clone transfected with wild-type Stat3 (clone B, see Fig 5B) displayed robust self-renewal in LIF + PD and expression of nuclear Stat3 targets (Appendix Fig S9A and B).

Thus, Stat3 specifically localized to the mitochondria is able to enhance transcription of mitochondrial genes and proliferation, but is unable to sustain ES cell identity.
Elena Carbognin et al
Stat3 activates mitochondria in pluripotent cells

The EMBO Journal
Vol 35 | No 6 | 2016

Figure 4.
LIF-dependent regulation of mitochondrial activity is critical for induction of naïve pluripotency

Signaling from LIF via Stat3 is important for the induction of naïve pluripotency. Over-activation of LIF/Stat3 is sufficient to reprogram EpiSCs to naïve pluripotent iPSCs (Han et al., 2010; Yang et al., 2010; Onishi et al., 2014). Expression of several transcription factors can also convert EpiSCs into naïve iPSCs (Guo et al., 2009; Hall et al., 2009; Hanna et al., 2009; Silva et al., 2009; Han et al., 2010; Festuccia et al., 2012; Gillich et al., 2012; Martello et al., 2013), but the presence of LIF invariably enhances the efficiency of conversion. The preceding results provoked the suggestion that LIF may exert functions during reprogramming beyond rewiring of the transcription factor network controlling pluripotency. During reprogramming of EpiSCs, mitochondrial respiration must be actively boosted to the level of naïve pluripotent cells (Zhou et al., 2012). We hypothesized that LIF could contribute directly by promoting mitochondrial transcription and activity.

We confirmed that EpiSCs have a greatly reduced OCR compared with ES cells (Appendix Fig S10A). We also found a general reduction in expression of known nuclear and mitochondrial Stat3 targets in EpiSCs (Fig 6A). To examine the involvement of mitochondrial respiration in the reprogramming process, we took advantage of the GOF-18 EpiSC line (Han et al., 2010). A fraction of GOF18 EpiSCs exhibit spontaneous conversion in 2i after 48 h exposure to LIF (Han et al., 2010; Yang et al., 2010). When the Complex I inhibitor rotenone was added together with LIF, we observed a severe reduction in the yield of IPS cell colonies (Fig 6B and C). Similar results were obtained in a second EpiSC line (Appendix Fig S10B) in which resetting to naïve pluripotency is driven by transient hyperactivation of Stat3 (Yang et al., 2010). We also exposed cells to rotenone 4 days after LIF induction and observed no difference in the number of IPS colonies obtained (Fig 6D and Appendix Fig S10C). Importantly, the low doses of rotenone used are tolerated well by both EpiSCs and ES cells (Appendix Fig S10D-G and Fig 4A and G), suggesting that the reduction in colony number is not due to toxicity. IPS cells obtained either in the presence or in the absence of rotenone treatment could self-renew in 2i + LIF without feeders over multiple passages and showed reactivation of naïve markers and shutdown of EpiSC markers (Fig 6E), suggesting that they are bona fide naïve pluripotent cells. These results suggest that upregulation of mitochondrial respiration is specifically required during the first 4 days of reprogramming.

To elucidate further the molecular mechanism underlying the effect of LIF and rotenone on reprogramming, we inspected the mitochondrial and nuclear transcriptional targets of Stat3 and observed that 48 h of LIF treatment in 2i is sufficient to induce both classes of gene in GOF18 EpiSCs (Fig 6F and G, compare blue and gray bars). The upregulation of mitochondrial targets indicates that Stat3 is active in the mitochondria in EpiSCs. All Stat3 targets are induced at the same levels, either in the presence or in the absence of rotenone treatment could self-renew in 2i + LIF without feeders over multiple passages and showed reactivation of naïve markers and shutdown of EpiSC markers (Fig 6E), suggesting that they are bona fide naïve pluripotent cells. These results suggest that upregulation of mitochondrial respiration is specifically required during the first 4 days of reprogramming.

Our findings suggest that LIF activates two programs, a nuclear program promoting rewiring of the transcription factor network and a mitochondrial program important for resetting the metabolic profile of the cell. To deconvolute the relative contributions of each program, we expressed two critical nuclear targets, Klf4 and Tcf3p21 (Niwa et al., 2009; Martello et al., 2013; Ye et al., 2013), in EpiSCs. Either factor, or the combination of the two together, was sufficient to reprogram EpiSCs without LIF consistent with previous reports (Yang et al., 2010; Martello et al., 2013), but in all cases the
Figure 5. Elena Carbognin et al
Stat3 activates mitochondria in pluripotent cells

The EMBO Journal
Vol 35 | No 6 | 2016

Published online: February 22, 2016
The EMBO Journal
Vol 35 | No 6 | 2016

© 2016 The Authors

The EMBO Journal
Vol 35 | No 6 | 2016

627
Stat3 activates mitochondria in pluripotent cells

Elena Carbognin et al

Figure 5. Mitochondrial Stat3 enhances the transcription of mitochondrial genes and proliferation of ES cells.

A Experimental approach used to characterize the functional role of Stat3 on cell proliferation and mitochondrial activity.
B Western blot of Stat3+/+ cells cultured in the presence or absence of LIF, Stat3−/− cells cultured in 2i + LIF, and two clones of Stat3−/− cells transfected with a full-length form of Stat3 cultured in 2i + LIF. Relative mean intensity is shown below each band. Note that Stat3 protein levels in clone A and B are comparable to the endogenous levels of the control. GAPDH was used as a loading control.
C Gene expression analysis of Stat3+/+ cells, Stat3−/− cells, and two Stat3 rescue clones (Stat3A/B) cultured in the presence or absence of LIF. Note that both clones respond to LIF and activate Stat3 direct target Socs3.
D Proliferation assay of Stat3+/+ cells, Stat3−/− cells, and Stat3A/B rescue clones cultured in the presence of LIF. Cells were seeded and scored for 4 days. Scores were normalized to day 1. Mean and s.e.m. of two independent biological replicates of a representative experiment are shown. See also Appendix Fig S7A.
E Western blot of total and mitochondrial fractions of Stat3+/+, Stat3−/− cells, and two MLS-Stat3 clones cultured in 2i + LIF. The nuclear protein TRIM33 and mitochondrial marker TOM20 confirmed successful mitochondrial isolation. Note that MLS-Stat3 is enriched in the mitochondrial fraction, suggesting correct localization of the fusion protein. See also Appendix Fig S7B.
F Representative confocal images of Stat3+/− and MLS-Stat3 cells stained with anti-Stat3 and anti-Atad3 antibodies. Merge shows colocalization between Stat3 and the nucleoids marker Atad3 (R = 0.72 for MLS-Stat3 cells, R = 0.22 in Stat3−/−). DAPI serves as a nuclear counterstain. Scale bar, 10 μm.
G Gene expression analysis of Stat3+/+, Stat3−/− cells, and three MLS-Stat3 clones cultured in the presence of LIF. MLS-Stat3 specifically induces expression of mitochondrial markers with negligible effects on the nuclear target Socs3. Mean and s.d. of two technical replicates. See also Appendix Fig S7E.
H Chromatin immunoprecipitation (ChIP) performed using anti-Stat3 or a rabbit control IgG antibody in Stat3+/− and MLS-Stat3 cells cultured in 2i + LIF conditions. ChIP-PCR was performed with primers located on three regions of the D-loop (A, B, C). Note that 2 D-loop regions are significantly enriched in MLS-Stat3 compared to Stat3−/− cells. Mean and s.e.m. of three independent experiments are shown. Unpaired t-test: *P < 0.05, **P < 0.01.
I Left: representative confocal images of Stat3+/− and MLS-Stat3 cells subjected to proximity ligation assay (PLA) by using anti-Stat3 and anti-Atad3 antibodies. DAPI serves as a nuclear counterstain. Red dots indicate spacial proximity between Stat3 and the nucleoids marker Atad3. Right: histogram showing quantification of PLA performed on Stat3+/+, Stat3−/−, and MLS-Stat3 cells. A number of red dots/cell are plotted. Note that double staining in MLS-Stat3 cells results in increased number or red dots compared to Stat3−/− cells. Mean and s.e.m. of ~15 cells for each sample are shown. Unpaired t-test: **P < 0.01, ***P < 0.001. Scale bar, 10 μm. See also Appendix Fig S7D.
J Proliferation assay of Stat3+/+, Stat3−/− cells, and three MLS-Stat3 clones cultured in the presence of LIF. Cells were seeded and scored for 4 days. Mean and s.e.m. of two technical replicates of a representative experiment are shown. See also Appendix Fig S8A.
K Proliferation assay of Stat3+/− cells and three MLS-Stat3 clones cultured in the presence of LIF. Cells were seeded and treated with DMSO or with 50 or 100 nM of rotenone for 48 h. MLS-Stat3 clones also proliferated more than Stat3−/− both basally and in the presence of rotenone. Mean and s.e.m. of two technical replicates of a representative experiment are shown.
L Representative bright field images of Stat3+/+, Stat3−/−, and one MLS-Stat3 clone cultured in 2i + LIF showing similar morphology, but note smaller colony size for Stat3−/− cells. Scale bar, 100 μm.

Source data are available online for this figure.
Figure 6.
activity is reduced, but not ablated in ES cells. Interestingly, glycolysis does not appear to be affected by LIF/Stat3 activation. In particular, we did not observe a compensatory increase in glycolysis when mitochondrial respiration was reduced, even though cells are cultured in high concentrations of glucose (Appendix Fig S3B and C). This may suggest that glycolysis serves as the basal energy source and mitochondrial respiration is used as “spare capacity” that can be enhanced by LIF/Stat3.

Activating mitochondrial respiration represents a potential roadblock in the induction of naïve pluripotency. In this context, the transcription factor network controlling pluripotency operates in tandem with metabolic regulation. Thus, when pluripotency factors are expressed in EpiSCs, LIF further enhances reprogramming in a manner dependent on mitochondrial respiration. Conversely, chemical blockade of mitochondrial respiration does not affect the expression of pluripotency markers. This result is consistent with several reports showing that inhibition of LIF signaling, via Jak inhibitor or genetic inactivation of Stat3, potently reduces or abolishes reprogramming to naïve pluripotency, even if critical factors such as Oct4, Sox2, and Klf4 are over-expressed (Yang et al., 2010; van Oosten et al., 2012; Tang et al., 2012).

Our study links the LIF/Stat3 axis directly to mitochondrial activity, but we still do not know how the levels of mitochondrial respiration affect ES cell proliferation or EpiSC resetting. Preliminary evidence would suggest that this is not simply due to altered ATP production. It will be interesting to investigate how LIF/Stat3 affects the global metabolic profile of ES cells in order to identify specific pathways involved in increased proliferation. Several recent studies have highlighted the potential crosstalk between metabolism and epigenetic modifications in pluripotent cells (Lu & Thompson, 2012; Carey et al., 2015). The action of LIF/Stat3 on mitochondria may therefore be important not to fulfill a bioenergetic requirement, but to increase the production of cofactors for epigenetic processes, such as demethylation of DNA and histone modification.

Materials and Methods

Embryonic stem cell culture

ESCs were cultured without feeders on plastic coated with 0.2% gelatine (Sigma, cat. G1890) and replated every 3–4 days at a split ratio of 1:10 following dissociation with Accutase (GE Healthcare, cat. L11-007). Cells were cultured either in serum-free N2B27-based medium (DMEM/F12 and Neurobasal [both Life Technologies] in 1:1 ratio, 0.1 mM 2-mercaptoethanol, 2 mM l-glutamine, 1:200 N2 [Life Technologies], and 1:100 B27 [Life Technologies]) supplemented with small-molecule inhibitors PD (1 μM, PD0325901), CH (3 mM, CHIR99021) from Axon (cat. 1386 and 1408) and LIF (100 units/ml produced in house), or in GMEM (Sigma, cat. G5154) supplemented with 10% FBS (Sigma, cat. F7524), 100 mM 2-mercaptoethanol (Sigma, cat. M7522), 1× MEM non-essential amino acids (Invitrogen, cat. 1140-036), 2 mM l-glutamine, 1 mM sodium pyruvate (both from Invitrogen), and 100 units/ml LIF.

EpiSCs were cultured without feeders on plastic coated with fibronectin (Millipore, cat. FC010) and replated every 2 days at a
split ratio of 1:10 following dissociation with Dispase (Stem Cell Technologies, cat. 07923). Cells were cultured in serum-free media N2B27 (see above) supplemented with FGF2 (12 ng/ml) and activin (20 ng/ml) produced in house. Oct4-GFP (OE2 Y118 line) was described in Yang et al (2010). GOF18 Episcs were described in Han et al (2010) and generously provided by Hans Schöler.

For DNA transfection, we used Lipofectamine 2000 (Life Technologies, cat. 11668-019) and performed reverse transfection. For one well of a 6-well plate, we used 6 µl of transfection reagent, 2 µg of plasmid DNA, and 300,000 cells in 2 ml of N2B27 medium. The medium was changed after overnight incubation.

Stable transgenic ESCs lines expressing Stat3 or MLS-Stat3 were generated by transfecting cells with PiggyBac transposon plasmids CAG-Stat3 or CAG-MLS-Stat3 with piggyBac transposase expression vector pBac. Selection for transgenes was applied, and stable clones were selected in 2i conditions.

For LIF induction experiments, ES cells were cultured in 2i without LIF for > 2 passages, plated (8,000 cells/cm²) in 2i. Twenty-four hours after plating, cells were treated with LIF for the indicated amount of time.

For AP staining, cells were fixed with a citrate-acetone-formaldehyde solution and stained using the Alkaline Phosphatase kit (Sigma, cat. 86R-1KT). Plates were scanned using a Nikon Scanner and scored manually.

RNA-seq data analysis
RNA sequencing data used in this study are described in Martello et al (2013) and are available in the ArrayExpress repository under accession E-MTAB-1796.

Proliferation assay
Cell proliferation was assessed by plating 15,000 ES cells in 12-well plate. Cells were counted every 24 h for 4 days. For rotenone (Sigma, cat. R8875), antimycin A (Sigma, cat. A8674), and myxothiazol (Sigma, cat. T5580) treatments, cells were plated in the presence of the inhibitors and scored after 48 h.

Cell cycle analysis
Cell cycle analysis was performed by staining single live cells with propidium iodide (Sigma, cat. P4170), according to the manufacturer’s instructions. Samples were analyzed by flow cytometry using a BD FACSCanto™ cytometer.

ROS measurement assay
Reactive oxygen species production was detected by staining single live cells with 2,7’-dichlorodihydrofluorescein diacetate (H2DCFDA) (Life Technologies, cat. D999), according to the manufacturer’s instructions. Samples were analyzed by flow cytometry using a BD FACSCanto™ cytometer.

Reprogramming assay
Episcs lines bearing an Oct4-GFP reporter were plated in Fgf2/activin medium and switched to 2i + LIF (with or without rotenone) conditions the next day. Human LIF was used at a concentration of 20 ng/ml. Cells were kept in 2i + LIF medium for 5 days, if not indicated otherwise, before switching to 2i. Reprogramming experiments were ended 6/8 days after medium switch to 2i/LIF, and Oct4-reporter-positive iPsc colonies were scored manually.

Propidium iodide/annexin V staining
PI/AnnV staining was performed on live single ESCs or Episcs according to the manufacturer’s instructions (Ebioscience, ref. 88-8007-72). Samples were analyzed by flow cytometry using a cytometer BD FACS Canto™ with BD FACSDiva™ software.

Flow cytometry
After treatment with Accutase (GE Healthcare, cat. L11-007), dissociated ESCs were resuspended in PBS. Flow cytometry analyses were performed using a cytometer BD FACS Canto™ with BD FACSDiva™ software.

Mitochondria isolation
Mitochondria isolation was performed from 4 × 10⁷ cells pellet as previously described in Frezza et al (2007). For BNGE analysis, mitochondria were isolated as described in Cogliati et al (2013).

Oxygen consumption assay
Oxygen consumption was measured using the Seahorse XF24 (Seahorse Bioscience). For this, ~20 h before the analysis Stat3+/+ and Stat3−/− cells were seeded in a 24-well cell culture plate (Seahorse Bioscience) coated with laminin (Sigma, cat. L2020) at a density of 140,000 cells per well in N2B27 media supplemented with 2i or 2i + LIF (as indicated). It is crucial to have an evenly plated monolayer of cells to obtain reliable measurements. Cells were maintained in a 5% CO₂ incubator at 37°C, and 1 h before the experiment, the cells were washed and incubated in 600 µl of DMEM containing 10 mM glucose (DMEM-high glucose) pH 7.4 at 37°C in a non-CO₂ incubator.

During the experiment, oxygen concentration was measured over time periods of 2 min at 5-min intervals, consisting of a 3-min mixing period and 2-min waiting period. Measurements of OCR in basal conditions were used to calculate the basal mitochondrial respiration. After this, the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) was added into the media at a final concentration of 200 nM. Oxygen consumption during this phase reflects the maximal mitochondrial respiratory capacity. Finally, ETC activity was blocked by the addition of rotenone or antimycin A, both at a final concentration of 200 nM. As a result, OCR drops dramatically, and the oxygen consumed in this situation by the cells comes from a non-mitochondrial origin.

Gene expression analysis by quantitative PCR with reverse transcription
Total RNA was isolated using RNaseasy kit (QIAGEN), and complementary DNA (cDNA) was made from 1 µg using M-MLV Reverse
Transcriptase (Invitrogen) and dN6 primers. For real-time PCR, we used SYBR Green Master mix (Bioline. Cat. BIO-94020). Primers are detailed in Table 1. Technical replicates were carried out for all quantitative PCR. An endogenous control (beta-actin) was used to normalize expression.

Luciferase assay

Luciferase reporter plasmid was derived by subcloning of the D-loop promoter region into pGL3-basic luciferase plasmid (Addgene). CMV-lacZ has been previously described in Lukas et al. (1997).

Embryonic stem cells and EpiSCs were plated in a 12-well plate and transiently transfected with luciferase reporter plasmid with CMV-lacZ to normalize for transfection efficiency (based on CPRG and transiently transfected with luciferase reporter plasmid with Luci ferase activity was determined in a Tecan plate luminometer with hours after transfection, the cells were harvested in Luc lysis buffer (25 mM Tris pH 7.8, 2.5 mM EDTA, 10% glycerol, 1% NP-40). Luciferase activity was determined in a Tecan plate luminometer with freshly reconstituted assay reagent (0.5 mM D-luciferin, 20 mM tricine, 1 mM (MgCO3)2Mg(OH)2, 2.7 mM MgSO4, 0.1 mM EDTA, 33 mM DTT, 0.27 mM CoA, 0.53 mM ATP).

Immunoblotting

Immunoblotting was performed as previously described in Yang et al. (2010). For BNGE, immunoblotting was performed as in Cogliati el al (2013). For antibodies details, see Table 2. Images were digitally acquired using a ImageQuant LAS4000 (GE Healthcare).

Immunofluorescence

For immunofluorescence, cells were fixed for 10 min in cold methanol at –20°C, washed in TBS, permeabilized for 10 min with TBST + 0.3% Triton X-100 at RT, and blocked for 45 min in TBS + 3% goat serum at RT. The cells were incubated overnight at 4°C with primary antibodies. After washing with TBS, the cells were incubated with secondary antibodies (Alexa, Life Technologies) for 30 min at RT.

Cells were mounted with ProLong® Gold Antifade Mountant with DAPI (Life Technologies, cat. P36941). Images were acquired with a Leica SP2 confocal microscope equipped with a CCD camera. For antibodies used, see Table 2. We quantified the degree of colocalization between different proteins by calculating the Pearson’s coefficient R by using the “coloc2” function of the freely available software Fiji (http://fiji.sc/Fiji).

Proximity ligation assay (PLA)

Proximity ligation assay was performed after an overnight incubation with primary antibodies following the manufacturer’s instructions (Olink Bioscience). Images were acquired with a Leica SP5 confocal microscope equipped with a CCD camera. Images acquired were analyzed using a custom macro for ImageJ, allowing automated and unbiased analysis.

### Table 1. PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mNd1</td>
<td>ccaccttacgctgcatgcc</td>
<td>atgctgatctagccaaaaat</td>
</tr>
<tr>
<td>mNd4</td>
<td>cgccctacccgttaggtc</td>
<td>gtgagcccatgctgattat</td>
</tr>
<tr>
<td>mNd4l</td>
<td>ctcctactcatgtaacctc</td>
<td>ggtcgcgaatctaaagatg</td>
</tr>
<tr>
<td>mCo3</td>
<td>tacccttggcttacctaatc</td>
<td>atagagctgtgctggcttg</td>
</tr>
<tr>
<td>mPouf5</td>
<td>gtttgagaggggtgaaacca</td>
<td>cttcgctcagggctcttc</td>
</tr>
<tr>
<td>mSot2</td>
<td>ccaactctgggatgcaacaa</td>
<td>tctggctcagcacaagat</td>
</tr>
<tr>
<td>mNanog</td>
<td>ttctttcctaaagggtgcg</td>
<td>aaggggagggcagggaga</td>
</tr>
<tr>
<td>mEsrb</td>
<td>gggtctctgtaaggaaccaaca</td>
<td>cccactttaggacatctcat</td>
</tr>
<tr>
<td>mXif4</td>
<td>cgggagggaggagaacact</td>
<td>gatgctcctagcggcaac</td>
</tr>
<tr>
<td>mTfcp2l1</td>
<td>ggggcattcagctggctact</td>
<td>ttcgcagatcctccttg</td>
</tr>
<tr>
<td>mSocs3</td>
<td>atttcgtgcgggagcatc</td>
<td>aactctgtgqgggagcat</td>
</tr>
<tr>
<td>mStat3</td>
<td>ttggagccgacatcttccag</td>
<td>gaggctcttcacacctctca</td>
</tr>
<tr>
<td>mRex1</td>
<td>ttctttcctaataggtgagtgqgtgc</td>
<td>gttcttccttggctgaggga</td>
</tr>
<tr>
<td>mFgf5</td>
<td>aaccctcgactgaaggcaaat</td>
<td>ccagggcatgtgattatagctg</td>
</tr>
<tr>
<td>mLevy1</td>
<td>ccacccgcaacgctcctaat</td>
<td>gcgcagaagcaacaaaatctgt</td>
</tr>
<tr>
<td>mBactin</td>
<td>ctaagcccaacgtggaaggaag</td>
<td>acacaggggacatacgagga</td>
</tr>
<tr>
<td>mLfut53</td>
<td>ttatgtgcctagggagcatc</td>
<td>aatcttggccagcctcact</td>
</tr>
<tr>
<td>ChiP A</td>
<td>cattaactatttttctctgaaca</td>
<td>caatgggtggaagggatagt</td>
</tr>
<tr>
<td>ChiP B</td>
<td>aatgcgtggcactgccctac</td>
<td>ttcctcagcttaggpcctct</td>
</tr>
<tr>
<td>ChiP C</td>
<td>tegtcgcaaacaacactca</td>
<td>ttagctaggcagcatagttgga</td>
</tr>
</tbody>
</table>

### Table 2. Antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Stat3</td>
<td>Mouse monoclonal</td>
<td>Cell Signalling cat. 9139</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-pStat3 (Y705)</td>
<td>Rabbit monoclonal</td>
<td>Cell Signalling cat. 91455</td>
<td>WB: 1:2,000</td>
</tr>
<tr>
<td>anti-TOM20</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnologies cat. 11415</td>
<td>WB: 1:2,000</td>
</tr>
<tr>
<td>anti-TIMM23</td>
<td>Mouse monoclonal</td>
<td>BD Biosciences cat. 61223</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-TRIM33</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotechnologies cat. 101179</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-laminB</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotechnologies cat. 6216</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-NDUF8B</td>
<td>Mouse monoclonal</td>
<td>Abcam cat. AB110242</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-ATP synthase</td>
<td>Mouse monoclonal</td>
<td>Abcam cat. AB14748</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-GAPDH</td>
<td>Mouse monoclonal</td>
<td>Millipore cat. MAB374</td>
<td>WB: 1:1,000</td>
</tr>
<tr>
<td>anti-SH3A</td>
<td>Mouse monoclonal</td>
<td>Abcam cat. Ab14715</td>
<td>WB: 1:2,000</td>
</tr>
<tr>
<td>anti-Ala3A</td>
<td>Rabbit monoclonal</td>
<td>AB-Biotecnologies cat. 224485</td>
<td>IF: 1:100</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>Mouse monoclonal</td>
<td>Progen cat. 61014</td>
<td>IF: 1:1,000</td>
</tr>
</tbody>
</table>
Chromatin immunoprecipitation (ChIP)

For ChIP experiments, cells were crosslinked, lysed, and sonicated as described in Enzo et al. (2015). For immunoprecipitation, sheared chromatin from 5 × 10^6 cells was incubated overnight at 4°C with 3 μg of rabbit monoclonal anti-Stat3 (Santa Cruz Biotechnologies, cat. sc-482) or with control rabbit IgG. Protein A Dynabeads (Life Technologies) were added for 3 h after extensive blocking in 0.5% BSA. Washing, de-crosslinking, and DNA purification were performed as in Enzo et al. (2015). Results were analyzed by qPCR. Since the D-loop region is partially duplicated in the nuclear genome, we designed primers specific for the mitochondrial genome (see Table 2).

Data availability


Expanded View for this article is available online.

Acknowledgements

We thank members of the Smith laboratory and the Martello laboratory for advice and discussion. We are grateful to Sinio Dupont, YaoYao Chen and Christian Frezza for critical reading of the manuscript and to Irene Zorzan, Marco Sciacovelli, and Valentina Giorgio for technical support. GM’s laboratory is supported by grants from Giovanni Armenise-Harvard Foundation and Telethon Foundation (TCP13013). The Cambridge Stem Cell Institute receives core funding from the Wellcome Trust and Medical Research Council. GM was supported by a Human Frontier Science Program Fellowship. AS is a medical research professor.

Author contributions

GM and AGS designed the study. GM, EC, and RMB carried out, analyzed, and interpreted experiments. MES performed BNGE assays, provided reagents and technical support. GM and AS supervised the study and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References


Tai C-I, Ying QL (2013) Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state. J Cell Sci 126: 1093–1098