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Abstract

Naive pluripotent epiblast cells of the preimplantation murine embryo and their *in vitro* counterpart, embryonic stem (ES) cells, have the capacity to give rise to all cells of the adult. Such developmental plasticity is associated with global genome hypomethylation. However, it is still unclear how genome methylation is dynamically regulated. Here we show that LIF/Stat3 signaling induces genomic hypomethylation via metabolic reconfiguration. In Stat3-/- ES cells we observed decreased alpha-ketoglutarate (αKG) production from reductive Glutamine metabolism, leading to increased Dnmt3a/b expression and to a global increase in DNA methylation. Notably, genome methylation is dynamically controlled by simply modulating αKG availability, mitochondrial activity, or Stat3 activation in mitochondria, indicating effective crosstalk between metabolism and the epigenome. Molecularly, αKG reduces expression of Otx2 and its targets Dnmt3a/b. Genetic inactivation of Otx2 or Dnmt3a/b results in genomic hypomethylation even in the absence of active LIF/Stat3, while Tet1/2 inhibition was inconsequential. Stat3-/- ES cells also show increased methylation at Imprinting Control Regions accompanied by differential expression of cognate imprinted transcripts. Single-cell transcriptome analysis of Stat3-/- embryos confirmed the dysregulated expression of Otx2, Dnmt3a/b and imprinted genes in vivo. Our results reveal that the LIF/Stat3 signal bridges the metabolic and epigenetic profiles of naive pluripotent cells, ultimately controlling genome methylation via Dnmt3a/b regulation. A wide range of cancers displays Stat3-overactivation and abnormal DNA methylation, raising the possibility that the molecular module we described here is exploited under pathological conditions.

Main Text:

After fertilization, the zygotic genome is demethylated in order to establish a blank canvas for embryonic development. DNA methylation occurs on carbon 5 of cytosine (5mC) and is catalyzed by DNA methyltransferases (DNMTs). Ten-eleven translocation (TET) proteins promote oxidation of 5mC to hydroxymethylcytosine (5hmC -[1,2]). Additional oxidation steps mediated by TETs lead to the conversion of 5hmC into unmodified cytosine. DNMTs and TETs are dynamically expressed during early development, leading to a local minimum of 5mC at the pre-implantation blastocyst stage at E3.5[3–5]. Imprinted genes, expressed monoallelically in a parent-of-origin fashion, resist this wave of DNA demethylation. Such monoallelic expression allows tight control of their dosage and is essential for the proper development of the embryo[6].

How is the expression of Dnmts and Tets controlled in the early embryo? In the embryo, the Jak/Stat pathway is active from E2.5 and E3.5, as shown by phosphorylation of Stat3 and transcriptional activation of its targets Socs3 and Tfcp2l1[7–11]. Thus, Stat3 could represent a good candidate as a regulator of Dnmt and Tet expression.

Mouse embryonic stem (ES) cells are in a naïve pluripotent state and share distinguishing molecular features with the preimplantation epiblast[12]. In particular, mES cells show genomic hypomethylation, similarly to the E3.5 blastocyst, but only when cultured in the presence of LIF, a ligand of the Jak/Stat pathway, in combination with two kinase inhibitors of GSK3 and MEK (2iLIF conditions -[13–17]). Such hypomethylation was attributed to the downregulation of Dnmt3a/b by the MEK inhibitor[14–17]. Conversely, mES cells cultured in fetal bovine serum-based medium with LIF (Serum LIF conditions - [18]), display higher levels of DNA methylation. Such findings indicate that LIF is not sufficient to induce genomic

hypomethylation in presence of serum, but the requirement of LIF or Stat3 to induce hypomethylation in 2iLIF conditions has never been formally tested.

Stat3 may represent an ideal regulator of the epigenome, considering its capacity to regulate gene expression in the nucleus, together with the ability to control cellular metabolism in mitochondria by promoting oxidative phosphorylation (OXPHOS - [19–21]). Several metabolites are known as regulators or cofactors of enzymes catalyzing epigenetic modifications [22]. For all these reasons, we genetically tested the role of LIF/Stat3 axis on genome methylation of naive pluripotent cells.

Results:

LIF/Stat3 induces hypomethylation in mES cells via Dnmt3a/b regulation

We measured the levels of 5mC by quantitative immunostaining in mES cells and observed a strong decrease in signal intensity in 2iLIF compared to Serum LIF (Fig. 1a), as previously reported[14–17]. We then asked whether LIF and its downstream mediator Stat3 could be required for the decrease in 5mC. Wild-type (S3+/+) mES cells stably expanded in 2i, or Stat3-/- (S3-/-) cells in 2iLIF showed significantly higher levels of 5mC than S3+/+ in 2iLIF, comparable to those of S3+/+ cells in Serum LIF (Fig. 1a). We performed Mass Spectrometry in order to unequivocally identify global 5mC and unmethylated cytosine and confirmed that only S3+/+ cells in 2iLIF showed a reduced fraction of methylated cytosine (Fig. 1b). We further confirmed our findings by Reduced Representation Bisulfite Sequencing (RRBS, Fig. 1c and S1a). We conclude that active LIF/Stat3 signaling is required, in combination with 2i, to induce genomic hypomethylation.

We then asked how LIF/Stat3 could regulate the levels of 5mC. We measured the expression levels of factors involved in 5mC deposition, maintenance and oxidation and observed that S3+/+ cells in 2iLIF showed reduced expression only of *de novo* methyltransferases Dnmt3a and Dnmt3b and increased expression of Tet2 compared to S3+/+ cells in 2i or to S3-/- cells (Fig. 1d), while the maintenance methylase Dnmt1 was not regulated by LIF. Similar effects of LIF were observed also in an independent mES cell line (Rex1-GFPd2-RGd2[23] Fig. S1b) and were confirmed at the protein level by both Western Blot (Fig. 1e) and Mass Spectrometry (Fig. 1f and S1c).

We then asked whether the hypomethylation induced by LIF was dependent on Dnmt3a/b.

We first confirmed in an independent wild-type mES cell line (E14IVc) that culture in 2i led to hypermethylation relative to 2iL (Fig. 1g). We then analyzed two independent mutant clones for each genotype of Dnmt3a KO, Dnmt3b KO, and Dnmt3a/b double KO (dKO) mES cells (Fig. S1d). When cultured in 2i without LIF, Dnmt3a KO and Dnmt3b KO cells displayed a partial reduction of 5mC relative to wild-type cells in 2i, while Dnmt3a/b dKO cells cultivated in 2i were hypomethylated (Fig. 1g). Mass Spectrometry (Fig. 1h) and RRBS (Fig. 1i and S1e) further confirmed that Dnmt3a/b dKO cells in 2i displayed DNA methylation levels even lower than wild-type cells in 2iL. We conclude that the levels of Dnmt3a/b dictate the DNA methylation status of naive ES cells in 2i. Such conclusions were further supported by the overexpression of Dnmt3a and Dnmt3b in S3+/+ cells in 2iLIF, which led to increased 5mC levels (Fig. S1f-g).

We also tested whether 5mC oxidases could have a role in the observed hypomethylation induced by LIF. Tet1 and Tet2 are both robustly expressed in mES cells in 2iL (Supplementary table 4) and have redundant functions[24]. Thus, we knocked down Tet1 and Tet2 simultaneously in S3+/+ 2iLIF and observed no significant changes of 5mC (Fig. S1h-i). We conclude that Tet proteins do not appear to regulate 5mC levels in 2iL.

Next, we wanted to study the dynamics of LIF-induced expression changes. We performed quantitative reverse-transcriptase PCR (RT-qPCR) on S3+/+ cells stably cultured in 2iLIF (Fig. 1j, 1st bars), or in 2i (2nd bars) or acutely stimulated with LIF for 1, 4, 24 and 48 hours, starting from 2i (3rd to 6th bars). We confirmed that LIF was able to repress Dnmt3a and Dnmt3b, however transcriptional changes were slow, requiring 24 hours to reach significance. Such results prompted us to study the dynamics of 5mC changes upon LIF stimulation. Cells stably cultured in 2i were exposed to LIF for 24 and 48 hours and analyzed by

immunostaining. We observed a mild decrease in 5mC levels after 24 hours, while at 48 hours the levels of 5mC were as low as those of cells stably cultured in 2iLIF (Fig. 1k). The slow kinetics observed would indicate that Stat3 does not directly repress the transcription of Dnmt3a/b. We interrogated available Stat3 ChIP-seq data and could not detect binding at enhancers or promoters of Dnmt3a/b[25]. Further, we expressed in S3-/- cells a Stat3 construct fused to an Estrogen Receptor domain (S3ER, Matsuda et al), which localizes to the nucleus and activates direct Stat3 targets Socs3 and Klf4 upon Tamoxifen treatment (Matsuda e Yang stat3 reprogramming and Fig. S2a-b). Dnmt3a and Dnmt3b expression were unaffected and, crucially, 5mC levels were unchanged (Fig. S2b-d), indicating the nuclear Stat3 is not sufficient to reduce DNA methylation levels in ES cells in 2i. These results indicate that LIF/Stat3 induces a hypomethylated genome state, characterized by low Dnmt3a and Dnmt3b and that such effects are not explained by a direct transcriptional mechanism.

Impact of Stat3 on DNA methylation and transcription

We then asked which genomic regions showed a DNA methylation profile dependent on Stat3. DNA methylation is particularly abundant at repetitive elements, but we could not observe differences in 5mC levels between S3+/+ and S3-/- cells in 2iLIF (Fig. S3a). Interrogation of RRBS data for S3+/+ and S3-/- cells in 2iLIF identifies 381,607 differentially methylated sites, with 98.7% of them displaying gain of methylation in S3-/- cells (Fig. 2a). We used H3K4me3 and H3K27ac profiles to identify promoters and enhancers in mES cells, and measured the levels of DNA methylation at both genomic features. We observed increased DNA methylation in S3-/- cells in 3.6% (323/8782) of promoters and 36.5% (621/1701) of enhancers (Y axes in Fig. 2b-c), while only 2 out of 8782 promoters and 1 out of 1701 enhancers showed decreased DNA methylation. We then intersected transcriptome data, comparing S3+/+ and S3-/- cells, and asked whether the gene associated with each

promoter or enhancer was differentially expressed. The gain of DNA methylation at promoters was associated with downregulation of cognate genes in 20.7% (67/323) of the cases and with upregulation in 8% (26/323) of cases (Fig. 2b). For enhancers, we observed 13.8% (86/621) and 6.7% (42/621) of cognate genes significantly downregulated or upregulated, respectively. Among significantly regulated genes we noticed the pluripotency factors Klf5 (Fig. 2g). We repeated the same analyses comparing S3+/+ in 2i and in 2iLIF (Fig. 2d-f, Fig. S3b-c) and obtained highly comparable results, clearly demonstrating that absence of LIF or of Stat3 had overlapping effects on the transcriptome (Fig. S3b) and 5mC profile of ES cells.

Given that Dnmt3a/b appeared functionally relevant for the regulation of 5mC levels downstream of LIF (Fig. 1g-i) we predicted similar changes in DNA methylation in response to LIF activation or upon Dnmt3a/b inactivation. We found that 98.95% (447,539 out of 452,310) of hypomethylated regions detected in wild-type ES cells in 2iL compared to 2i were also hypomethylated in Dnmt3a/b dKO cells compared to wild-type ES cells (Fig. 2h-i, Fig. S3d-f). Such results further indicate that Dnmt3a/b are epistatic to LIF/Stat3 for DNA methylation control.

Imprinted genes are organized in clusters, under the control of Differentially Methylated Regions (DMRs). Imprinted transcripts are expressed monoallelically in a parent-of-origin fashion, allowing precise regulation of their dosage[26,27]. S3+/+ cells cultured long term in 2iLIF showed low levels of DNA methylation at imprinted DMRs, as previously reported[14,28], while S3-/- cells retained higher levels of DNA methylation at 83.3% (20/24) of DMRs analyzed (Fig. 2j), despite the prolonged culture in the 2iLIF medium. These findings were independently validated by MeDIP-qPCR (Fig. 2k and S3h).

We checked whether aberrant DNA methylation at DMRs affected gene expression and observed that 37% (20/54) of imprinted transcripts expressed in mES cells were differentially expressed in S3-/- cells (Fig. 2l and Fig. S3i). Furthermore, the percentage of differentially expressed imprinted genes, without taking into account DNA methylation information, was 50.77% (33/65), while only 18.77% (2349/12510) of all expressed genes were differentially expressed, indicating that imprinted genes are specifically regulated by Stat3 (Fig. 2m and S3j, *P* value= 5.67 x 10-9, hypergeometric test).

We conclude that Stat3 regulates DNA methylation at promoters, enhancers, and imprinted DMRs, with a concomitant change in expression of a fraction of their associated transcripts.

Stat3 controls DNA methylation via metabolic regulation

We observed that active LIF/Stat3 signaling in presence of 2i controls 5mC levels and expression of Dnmt3a/b (Fig. 1) and that such effects are not explained by direct transcriptional regulation by Stat3 (Fig. S2), therefore we sought for alternative mechanisms. First, we thought that global 5mC levels could be affected by passive dilution occurring during genome replication. Given that the LIF/Stat3 axis promotes mES cells proliferation[19] we asked whether reduced 5mC in S3+/+ cells in 2iLIF could be due to enhanced proliferation. We tested this hypothesis by performing the EdU incorporation assay combined with 5mC immunostaining. For S3-/- cells in 2iLIF or S3+/+ cells in 2i, we observed that EdU positive cells that underwent genome replication within the last 4 hours showed an expected decrease in 5mC compared to EdU negative cells (Fig. S4a), which never reached the levels of S3+/+ in 2iLIF. Moreover, 5mC levels in S3+/+ cells in 2iLIF were only marginally affected by the EdU status, indicating that differences in cell proliferation can account only in part for the decrease in 5mC induced by LIF/Stat3.

We then hypothesized that LIF/Stat3 might control 5mC levels via regulation of mitochondrial activity, given that it has previously been reported that S3-/- cells display reduced mitochondrial OXPHOS[19–21]. We, therefore, tested whether 5mC levels are dependent on changes in OXPHOS. First, we treated S3+/+ cells with inhibitors of Complex I and III of the respiratory chain at concentrations that reduce OXPHOS in mES cells[19] and observed a strong increase in 5mC signal (Fig. S4b). Importantly, Dnmt3a/b KO cells did not show any significant increase in 5mC upon inhibition of the respiratory chain (Fig. S4c), further indicating that changes in 5mC are dependent on Dnmt3a/b in mES cells. Second, we expressed at endogenous levels a Stat3 construct targeted to mitochondria in S3-/- cells (Fig. S4d-g)[19,29]. The two clonal lines, called MitoS3.A and MitoS3.B, showed increased OXPHOS (Fig. S4h) and reduced 5mC (Fig. 3a-b) compared to parental S3-/- cells.
Given that expression of imprinted transcripts is tightly linked to 5mC levels on imprinted DMRs, we measured their expression in S3-/- and MitoS3 cells and found that 25 were differentially expressed (Fig. 3c), accounting for 38.46% of imprinted transcripts expressed in mES cells. We conclude that the hypomethylation observed in 2iLIF is linked to robust OXPHOS of mES cells.

Our results indicate that mitochondrial activity affects the methylation profile of the nuclear genome, which implies that the two organelles are able to communicate. We initially hypothesized that intracellular signaling molecules, such as Calcium ions or Reactive Oxygen Species (ROS) could be implicated, but we did not observe differences in their abundance between S3+/+ and S3-/- cells, nor effects upon blockade of such signals (data not shown). We then reasoned that mitochondrial activity could influence the abundance of metabolites, in particular those serving as donors, acceptors or cofactors of DNA methylation and oxidation[22]. Analysis of steady-state levels of metabolites revealed a decrease in Alpha-

Ketoglutarate (αKG) in S3-/- cells (Fig. 3d). We also noticed a strong reduction in Methionine levels in Stat3-/- cells, however, nor SAMe, which is the actual donor of methyl groups to DNA and histones, neither the enzymes involved in Methionine/SAMe metabolism, such as MAT2A, MAT2B (Methionine Adenosyltransferase 2A/2B), AHCY (Adenosylhomocysteinase) and MTR (Methionine Synthase) were changed between Stat3+/+ and -/-, indicating that Methionine/SAMe metabolism might not be involved in DNA methylation regulation downstream of Stat3 (Supplementary table 7).

We then asked what carbon source was preferentially used by mES cells for production of αKG and performed metabolic flux analysis with ^{13}C-labelled Glucose, Glutamine or Palmitate. As previously reported[30], we observed that Glutamine represents the main source for production (Fig. S5a). Glutamine is directly converted into Glutamate and αKG, which in turn enters the Tricarboxylic Acid Cycle (TCA) for energy production via oxidative metabolism. Analysis of specific isotopomers revealed a decrease in the oxidative Glutamine pathway and TCA activity in S3-/- cells (Fig. S5b-c, OAA M4 and Citrate M4), in line with impaired OXPHOS (Fig S4h). Alternatively, in the reductive Glutamine pathway, Glutamine is converted in the cytosol into TCA intermediates and Acetyl-CoA, which is diverted to fatty acid biosynthesis. Conversely, Oxaloacetate (OAA) obtained from cytosolic citrate cleavage is converted to Aspartate or Malate and then Pyruvate, which feeds the TCA either directly or via conversion into OAA by Pyruvate Carboxylase (PCX). S3-/- cells showed impaired glutamine reductive pathway. Specifically, we detected a strong decrease of cytosolic OAA M3 and mitochondrial OAA M2, Citrate M2 and M4, αKG M2 and M4 (Fig. 3e-f and S5b-c). These data are sustained by decreased expression of cytoplasmic isocitrate dehydrogenase 1 (IDH1, Fig. S5d). Finally, a mild increase of αKG M5 was found in S3-/- cells indicating impaired flux of glutamine carbons into oxidative and reductive pathways (Fig. 3f and S5b-c).

Based on our observations we hypothesized that robust αKG production from Glutamine is required for genome hypomethylation. We measured αKG levels in cells expressing Stat3 only in mitochondria. Both MitoS3.A and MitoS3.B clones showed elevated αKG levels, not significantly different from S3+/+ cells (Fig. 3g). Both clones also showed reduced 5mC (Fig. 3a-b), further indicating that elevated αKG levels correlate with reduced DNA methylation.

To functionally test our hypothesis, we cultured S3+/+ cells in 2iLIF in the absence of Glutamine. We first measured the endogenous levels of αKG in cells cultured in the absence of Glutamine and found it strongly reduced (Fig. 3h) and observed a robust increase in 5mC (Fig. 3i).

Next, we asked whether restoring endogenous αKG levels could result in reduced 5mC. We added back a cell-permeable form of αKG (DM-αKG) and we were able to reduce 5mC levels (Fig. 3h-i). Of note, DM-αKG has been reported to stabilize Hypoxia-inducible factor 1-alpha (Hif1a) by inducing a pseudohypoxic state[31], but this was not the case in mES cells (Fig. S5e).

In sum, our results indicate that efficient αKG production from Glutamine induces low methylation levels of the nuclear genome.

Alpha-Ketoglutarate regulates 5mC mainly via control of Dnmt3a/b levels

Next, we asked how αKG reduces 5mC levels in mES cells. AlphaKetoGlutarate functions as a cofactor for Tet oxidases[22,32,33], thus S3-/- cells might display increased 5mC due to reduced Tet activity. In addition, it has been recently reported that the abundance of αKG inversely correlates with Dnmt3a/b expression levels[32]. Thus, S3-/- cells might show increased expression of Dnmt3a/b and 5mC, as a consequence of reduced αKG levels. To investigate the relative contribution of these two possible mechanisms, we took advantage of our MitoS3

cells, where αKG levels were rescued to endogenous levels (Fig. 3g) and 5mC were decreased (Fig. 3a-b), with no potentially confounding effects from nuclear Stat3.

Elevated αKG levels or, more precisely, high αKG/succinate and αKG/Fumarate ratios are associated with increased Tet activity[30,33–35]. We found no significant differences in the αKG/Fumarate ration, while αKG/succinate ration appeared equally low in S3-/- and MitoS3 cells relative to S3+/+ cells (Fig. 4a). We then measured h5mC and 5mC and used their ration as a direct readout of Tets activity, which appeared low both in S3-/- and MitoS3.A/B compared to S3+/+ cells (Fig. 4b, S6a). Such results indicate that in S3-/- and MitoS3 cells Tets activity is similarly low, therefore it could hardly explain the differences in 5mC between S3-/- and MitoS3 cells (Fig. 3a-b). Such conclusions are in line with the lack of effect on 5mC observed upon Tet1/2 knockdown (Fig S1h-i).

Next, we measured mRNA and protein levels of Dnmt3a/b by RNAseq, RT-qPCR, Western Blot and Mass Spectrometry and found that both Dnmt3a/b mRNA and protein levels were reduced in MitoS3.A and MitoS3.B cells compared to S3-/- cells (Fig. 4c-e, Fig. S6b), indicating that αKG could repress Dnmt3a/b expression.

To directly test whether αKG negatively regulates Dnmt3a/b expression, we treated S3-/- cells with DM-αKG and observed a partial reduction of Dnmt3a/b expression (Fig. 4f) and of imprinted genes (Fig. 4g). Such partial effects are likely due to the fact that DM-αKG is unable to stably rescue endogenous αKG levels in S3-/- cells (Fig. S6c).

This set of experiments led us to conclude that αKG decreases 5mC levels via reduction of Dnmt3a/b expression, rather than by increasing Tets activity. Of note, such conclusions are in agreement with our genetic perturbations showing that Dnmt3a/b levels dictate 5mC abundance (Fig. 1g-i and S1f-g).

αKG regulates Dnmts expression via the transcription factor Otx2

We next decided to clarify the molecular mechanism of how αKG controls the expression of Dnmt3a/b. First, we have explored literature and we have analyzed a database ("NIA Mouse ES Cell Bank"[36–38]) reporting transcriptomic data of a large number of mouse ES cell lines, in which single transcriptional regulators were either induced or repressed. From this survey, we have identified 2 activators (Otx2, Sox1[39,40]) and 6 repressors (Klf4, Nanog, Prdm14, Tbx3, Tcea3, Tcl1[41–46]) of Dnmt3a/b.

We thus checked the expression levels of our candidate regulators in S3+/+ and S3-/- cells, and observed that the activators Otx2 and Sox1 were upregulated in S3-/- cells, while the repressors Klf4, Tbx3 and Tcl1 were downregulated in S3-/- cells (Fig. 4h). Given that mitochondrial Stat3 expression increased endogenous αKG levels, and reduced 5mC levels (Fig. 3a-b) and Dnmt3a/b expression (Fig. 4c-d), we measured the expression of Dnmt3a/b potential regulators in MitoS3 cells and observed that only Otx2, Klf4 and Tcl1 expression was significantly affected by mitochondrial Stat3 (Fig. 4h). Such results were confirmed by RNA-seq analysis (Fig. S6d). Finally, we asked whether αKG treatment would affect the expression of our putative Dnmt3a/b regulators, and we observed a significant effect only in the case of Otx2 (Fig 4i).

To test whether Otx2 is functionally required for Dnmt3a/b regulation we first cultured wild-type ES cells in 2i or 2iL. In the absence of LIF, Dnmt3a/b expression was increased together with Otx2 expression (~6 fold increase, Fig. 4j and Fig. S6e). This was accompanied by the expected increase in 5mC (Fig. 4k). We reasoned that if Otx2 is in fact crucial for Dnmt3a/b

regulation in this context, its genetic inactivation should have rendered cells unable to upregulate Dnmt3a/b in the absence of LIF. We cultured Otx2-/- ES cells[47] in 2i or 2iL and observed that Dnmt3a/b expression, as well as 5mC levels, were not significantly changed. We, therefore, conclude that Otx2 is regulated by the LIF/Stat3/αKG axis and that Otx2 is genetically required to boost Dnmt3a/b and 5mC levels in ES cells.

Mitochondrial Stat3 regulates the differentiation of ES cells.

Our results indicate that mitochondrial Stat3 regulates DNA methylation in naive ES cells. We then asked whether such regulation has any functional consequence on ES cell behavior. Mitochondrial Stat3 reduces the levels of Otx2, Dnmt3a/b and 5mC, which are all molecular markers of early phases of mES cell differentiation[4,23,48–54]. Therefore, we hypothesized that mitochondrial Stat3 might stabilize pluripotency and/or slow down differentiation.

To test this hypothesis, we generated transcriptomic data of cells either in 2iLIF or undergoing differentiation for 48 hours in the N2B27 basal medium. We first identified the genes significantly downregulated in S3-/- cells relative to S3+/+ in 2iLIF (Fig. 5a, blue), and observed that they were also downregulated during differentiation of S3+/+ cells (Fig. 5b). Several naive pluripotency markers belong to this category (Fig. 5d), including Esrrb, Klf5 and Tet2. Similarly, genes found upregulated in S3-/- cells relative to S3+/+ in 2iLIF (Fig. 5a, orange) were found upregulated in S3+/+ cells during differentiation (Fig. 5c). Among such genes, we found several early differentiation markers, such as Otx2, Lin28b and Pou3f2, as well as the imprinted genes Igf2, Sfmbt2, Cdkn1c and Phlda2 (Fig. 5e). Notably, S3-/- cells display a much faster reduction in naive markers and upregulation of early differentiation and imprinted genes after 48 hours in N2B27 (Fig. 5d-f and S7a-d). Furthermore, we performed a clonal assay of cells undergoing differentiation for up to 72 hours. In 2iLIF, the number of

Alkaline phosphatase positive (AP+) pluripotent colonies formed by S3-/- cells was mildly reduced relative to S3+/+ (Fig. 5g), indicating that clonogenicity of S3-/- cells is partially impaired. After 24 hours of 2iLIF withdrawal, S3-/- cells formed fewer AP+ colonies than 2iL, while S3+/+ cell actually display an increase in AP+ colonies. Only after 48h of 2iLIF withdrawal S3+/+ cells showed a mild reduction in AP+ colonies relative to 2iL, while S3-/- cells at the same time point completely lost the capacity to form AP+ colonies. Eventually, after 72 hours of 2iLIF withdrawal, both cell lines lost clonogenicity.
Based on the faster transcriptional changes and the faster loss AP+ colonies we conclude that S3-/- cells exit more rapidly from the naive pluripotent state.

Such results are consistent with the canonical role of LIF and nuclear Stat3 as a transcriptional inducer of naive factors described by several laboratories[9,11,55,56]. In other words, the effects of Stat3 on differentiation could be completely independent from its capacity to control αKG production and 5mC from the mitochondria. Thus, we asked whether the expression of Stat3 in the mitochondria would affect differentiation of S3-/- cells. Transcriptionally, we observed that genes downregulated in S3-/- cells were only mildly affected by mitochondrial Stat3 (Fig. 5b and d). Conversely, early differentiation marker and imprinted genes were strongly reduced in MitoS3.A/B cells (Fig. 5c, e-f). Similar results were obtained when we analyzed only genes associated with genomic features differentially methylated in S3-/- cells (Fig. S8a-d). Finally, in the clonal assays, mitochondrial Stat3 restored clonogenicity in 2iLIF and delayed the exit from naive pluripotency (Fig. 5g).
We conclude that in the absence of Stat3, genes associated with naive pluripotency are prematurely downregulated, while early differentiation genes are overactivated, leading to partially compromised pluripotency and accelerated exit from the naive state. Expression of mitochondrial Stat3 counteracts such effects, specifically by repressing early differentiation

genes, indicating that modulation of mES cell metabolism by mitochondrial Stat3 has functional consequences on naive pluripotent cell behavior.

Stat3 regulates Dnmts and imprinted transcripts in the early mouse blastocysts.

Based on our in vitro observations, we decided to test the function of Stat3 in the early mouse embryo. We focused our attention on the early blastocysts at E3.5, because at this stage Stat3 is active[7,8,10] and the genome is hypomethylated[4,5] in naive pluripotent cells of the inner cell mass (ICM). At E3.75, ICM cells are specified into pluripotent Epiblast cells (Epi) or into extraembryonic Primitive Endoderm cells (PrE).

Stat3 heterozygous mice were crossed and blastocyst embryos at E3.5 and E3.75 were flushed. Trophectoderm cells were removed by immunosurgery and used to genotype individual embryos. Single ICM, Epi and PrE cells were analyzed by RNA sequencing (Fig. 6a).

Such results indicate that Stat3-/- ICM cells might precociously activate the PrE expression program. To test this hypothesis, we generated a list of genes specifically expressed in PrE at E4.5 and found them upregulated in Stat3-/- ICM cells compared to Stat3+/+ ICM cells (Fig. S9a).

We then analyzed cells from E3.75 embryos and individual cells could be classified as Epiblast or PrE according to specific markers (Fig. S9b). We also observed a clear separation between Stat3+/+ and Stat3-/- cells (Component 2 in Fig. 6e).

In Stat3-/- embryos, Socs3, Tfcp2l1, Nanog and Tet2 were significantly reduced in Epiblast cells at E3.75 (Fig. 6f-g). Interestingly, markers of post-implantation epiblast, such as Utf1, Otx2[7,57]and Dnmt3a/b were also upregulated in Stat3-/- cells (Fig. 6g).

Gene lists associated with Epiblast at E5.5 and E6.5 were also upregulated specifically in E3.75 Epi Stat3-/-, while genes associated with E3.5 ICM were downregulated (Fig. S9c left panel), further indicating accelerated developmental progression.

We observed that Stat3 regulates imprinted transcripts in vitro (Fig. 2). We analyzed all imprinted transcripts expressed at E3.5 or 3.75 and observed a global deregulation (Fig. 6h). For instance, we observed anticipated expression of Mest and Sfmbt2 or reduced expression of Rhox5 and Pon2 (Fig. 6i).

These results indicate that Stat3 regulates expression of Otx2, Dnmt3a/b and imprinted transcripts in the preimplantation blastocyst, ultimately affecting the pace of developmental progression.

Discussion

Mouse ES cells cultured in 2iLIF display low levels of 5mC and Dnmt3a/b, similarly to naive pluripotent cells of the pre-implantation blastocyst-stage embryo, while cells in Serum LIF show elevated 5mC and Dnmt3a/b. Previous studies reported that the hypomethylation observed in 2iLIF was due to the presence of the MEK inhibitor, one of the two inhibitors used in 2i. MEK inhibition causes upregulation of Prdm14[17,43,44], which, in turn, represses Dnmt3a/b expression[14,15].

Our results indicate that also mitochondrial Stat3 is necessary for Dnmt3a/b downregulation in 2iLIF, given that Stat3 null cells in 2iLIF displayed high 5mC levels, despite the presence of the MEK inhibitor, and expression of a mitochondrially localized Stat3 construct is sufficient to reduce Dnmt3a/b and 5mC levels. We note that PRDM14 expression was not affected by Stat3 (Fig. 4), overall indicating that Stat3 and PRDM14 are two independent negative regulators of Dnmt3a/b, and they are both genetically required for hypomethylation in 2iLIF.

Previous studies linked the hypomethylation of naive ES cells in 2iLIF to reduced de novo DNA methylation activity[14,15,17,41,42,58], in agreement with our results, showing that genetic inactivation or over-expression of Dnmt3a/b resulted in reduced or increased 5mC levels, respectively. In contrast, the maintenance DNA methyltransferase Dnmt1 shows similar mRNA levels, protein levels and activity both in 2iLIF and Serum LIF[14,59,60], indicating that the hypomethylation in 2iLIF is not due to reduced Dnmt1 activity.

In a recent paper von Meyen and colleagues found that Uhfr1 is required for the specific methylation at H3K9me loci[61].

We here demonstrated that mitochondrial Stat3-dependent reduction of Dnmt3a/b expression and 5mC levels genome wide is crucial to control genome hypomethylation after prolonged 2iLIF culture.

In the embryo, Otx2 and Dnmt3a/b are expressed robustly only after implantation at E5.5[7], while Stat3 is active only in the pre-implantation blastocyst[7]. We showed that genetic inactivation of Stat3 leads to anticipated expression of Otx2 and Dnmt3a/b, altogether indicating that Stat3 is needed to temporally restrict the expression of the post-implantation transcriptional program. Stat3 null embryos fail soon after implantation[62]. It would be interesting to test whether such embryonic lethality is due to accelerated development of Stat3 embryos relative to maternal tissues.

Stat3 has been shown to act in different cellular compartments, as nucleus, mitochondria and the Endoplasmic Reticulum[20,21,63]. We showed that mitochondrial Stat3 is critical for repression of Dnmt3a/b, through the control of αKG levels in the cell. Previous work implicated Stat3 in the control of epigenetic modifications during somatic cell reprogramming[64,65]. It would be interesting to test whether this is due to the metabolic activity of mitochondrial Stat3.

The two inhibitors of Gsk3 and Mek have been involved in metabolic rewiring allowing efficient conversion of αKG into Glutamine in mouse ES cells[30]. Moreover, αKG production from Glutamine via Psat1 has been reported to decrease during ES cell differentiation[66]. Our results complement such studies, showing that Glutamine is a major source of αKG production and that Stat3 predominantly regulates the reductive Glutamine pathway, overall indicating that multiple metabolic pathways allow interconversion of αKG and Glutamine, in

line with the critical roles of both metabolites in fundamental processes such as proliferation, epigenetic regulations and differentiation[30,32,67].

Of note, Stat3-/- cells show impaired mitochondrial respiration, a condition associated with enhanced reductive Glutamine metabolism in cancer cells[68], suggesting that aberrant activation of Stat3, or its upstream kinases JAKs, observed in several types of cancers might have an impact on Glutamine metabolism under pathological conditions.

Stat3 regulates imprinted gene expression, which depends on DNA methylation. In ES cells this regulation is due to mitochondrial Stat3 and αKG availability. Importantly, Stat3 inactivation *in vivo* also results in global dysregulation of imprinted genes. We observed that several imprinted (e.g. Ddc, Gab1, Commd1, Cobl, Cd81) have been shown to regulate ES cell differentiation[69], suggesting that a balanced expression of imprinted genes could be critical for correct exit from naive pluripotency.

Long term culture of female murine ES cells in 2iLIF has been associated with decreased methylation at imprinted DMRs[15,16,70,71] and reduction of MEK inhibitor concentration allowed to maintain robust methylation at DMRs over extensive culture. Similarly, we showed that in the absence of Stat3 mES cells maintain high methylation levels at DMRs after over 20 passages in 2iLIF, suggesting that tuning LIF/Stat3 activation might be important for the generation and long-term expansion of pluripotent cells with intact imprinting information.

Naive ES cells are characterized by bivalent metabolism, a hypomethylated genome and high expression of specific transcription factors and epigenetic modifiers, such as Tet2. Upon differentiation, OXPHOS is decreased, genome methylation is increased, naive specific genes

are downregulated and early markers of differentiation are upregulated, including Dnmt3a/b. We propose a model whereby all these molecular processes are elegantly under the control of a single molecule, Stat3. While nuclear Stat3 directly induces and maintains the expression of naive pluripotency factors, mitochondrial Stat3 promotes OXPHOS and αKG production, genome hypomethylation and inhibition of early differentiation markers. Such model explains previous observations, such as the inability of MitoS3 by itself to maintain long term self-renewal[19] and will be useful to test the role of LIF/Stat3 in pluripotent cells of other species and during induction of pluripotency.

FIGURE 1

Fig. 1.LIF/Stat3 induced hypomethylation in mES cells via Tet2 and Dnmt3a/b regulation

a, Immunofluorescence on S3+/+ cells cultured in Serum LIF, 2i or 2iLIF and S3-/- cells in 2iLIF stained with anti-5mC antibody (5mC, 5-methylcytosine). Top: Violin plots show the distribution of fluorescence intensity of an average of 63 nuclei per sample, normalized to the mean intensity of S3+/+ 2iLIF. Boxplots show 1st, 2nd and 3rd quartile; Whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the 4 conditions in analysis. Scale bar: 20μm.

b, Mass Spectrometry showing percentages of 5mC in the DNA of S3+/+ cells cultured in Serum LIF, 2i or 2iLIF and S3-/- cells in 2iLIF. 5mC contents are expressed as the percentage of 5mC in the total pool of cytosine. Bars represent means and s.e.m of n = 3 different biological replicates for S3+/+ Serum LIF and S3+/+ 2iLIF and n = 4 different biological replicates for S3+/+ 2i and S3-/- 2iLIF shown as dots. *P* values calculated using two-tailed unpaired T-test.

c, Frequency of DNA methylation at CpG islands measured by RRBS in S3+/+ cells cultured in Serum LIF, 2i or 2iLIF and S3-/- cells in 2iLIF. Each boxplot indicates the 1st, 2nd and 3rd quartile of a biological replicate. Whiskers indicate minimum and maximum value. See also Fig. S1a.

d, Heatmap for 7 genes involved in DNA methylation (Dnmt3a, Dnmt3b, Dnmt1, Dnmt3l, Uhrf1) and methylcytosine oxidation (Tet1, Tet2). RNAseq data derived from S3+/+ and S3-/- cells, expanded in 2i media without LIF or treated with LIF; n = 2 biological replicates are reported for each condition. Expression levels were scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. See also Fig. S1b.

e, Western blot of S3+/+ cells cultured in 2i or 2iLIF (left). Two biological replicates were loaded for each condition, indicated as R1 and R2. *De novo* methyltransferases DNMT3A(92 and 130kDa) and DNMT3B(130kDa) were less abundant in cells treated with LIF, while TET2 was increased. As a control, cells cultured in 2i or 2iLIF were exposed to differentiation in the basal medium for 24h (right). As previously reported [17], DNMT3A/B

were upregulated, while TET2 (220kDa) was downregulated. LAMINB (74kDa) was used as a loading control. Two isoforms of DNMT3A (92 and 130kDa) were detected, as previously reported[72].

f, Volcano plot of proteomics data, depicting differences in protein abundances between S3+/+ 2i and S3+/+ 2iLIF cells. Each dot represents one protein. The x axis shows the fold change (FC) in protein abundance (measured as LFQ intensity, in log-scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Yellow and blue dots indicate respectively proteins that are less or more abundant (FC > 1 or FC < -1 respectively, *P* value < 0.05) in S3+/+ 2i cells with respect to S3+/+ 2iLIF cells. Similar effects were observed also in S3-/- cells (Fig. S1c). All proteomics data are in Supplementary table 7.

g, Immunofluorescence for 5mC on wild-type mES cell line E14IVc (E14) cultured in 2iLIF and 2i, and on Dnmt3a KO, Dnmt3b KO, and Dnmt3a/b double KO mES cells cultured in 2i without LIF. Two independent mutant clones for each genotype were validated by Western blot (Fig. S1d) and analysed. Top: Violin plots show the distribution of fluorescence intensity of an average of 82 nuclei per sample, normalized to the mean intensity of E14 2iLIF. Boxplots show 1st, 2nd and 3rd quartile; Whiskers indicate minimum and maximum values. Independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the 5 conditions in analysis. Scale bar: 20μm.

h, Mass Spectrometry showing percentages of 5mC in the DNA of E14 cells cultured in 2iLIF and 2i, and Dnmt3a/b double KO mES cells in 2i (two independent mutant clones, Dnmt3a/b dKO.A and Dnmt3a/b dKO.B). 5mC contents are expressed as the percentage of 5mC in the total pool of cytosine. Bars represent means and s.e.m. of n = 5 different biological replicates shown as dots. *P* values calculated using two-tailed unpaired T-test.

i, Frequency of DNA methylation at CpG islands measured by RRBS in E14 cells cultured in 2iLIF and 2i, and two clones of Dnmt3a/b double KO mES cells in 2i. Two biological replicates for each sample. Each boxplot indicates the 1st, 2nd and 3rd quartiles. Whiskers indicate minimum and maximum value.

j, Gene expression analysis by RT–qPCR of S3+/+ cells stably cultured in 2iLIF, in 2i or acutely stimulated with LIF or for 1h, 4h, 24h and 48h. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n = 4 independent experiments, shown as dots. *P* values are calculated using two-tailed unpaired T-test relative to 2i and are shown only when <0.05.

k, Immunofluorescence staining of 5mC in S3+/+ cells cultured stably in 2iLIF or in 2i, and after LIF addition (for 24 and 48 hours). Top: Violin plots show the distribution of fluorescence intensity of an average of 86 nuclei per sample, normalized to the mean intensity of S3+/+ 2iLIF. Boxplots show 1st, 2nd and 3rd quartile. Whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the 4 conditions in analysis. Scale bar: 20μm.

FIGURE 2

Fig. 2. Impact of Stat3 on DNA methylation and transcription

a, Volcano plot showing the significant differentially methylated CpG sites between S3-/- and S3+/+ cells (Q-value < 0.01, abs(average methylation difference) > 10%). The x and y axis represent the difference in methylation levels (reported as percentage) and the statistical significance (in log-scale) respectively. Red dots depict hyper-methylated sites in S3-/- cells, blue dots the hypo-methylated ones. The analysis confirmed the overall gain in DNA methylation levels in S3-/- samples, with 376,303 hyper-methylated CpG sites and only 5,304 hypo-methylated sites out of a total of 1,230,955 sites mapped.

b, Scatter plot showing the mutual changes in gene expression (RNAseq) and DNA methylation levels (RRBS) at active promoters between S3+/+ and S3-/- cells. For each gene, the x axis reports the fold change in gene expression and the y axis the average methylation difference; red dots indicate genes for which both changes reached statistical significance (adjusted *P* value < 0.01 for gene expression, Q-value < 0.01 for methylation difference). See also Supplementary table 5.

c, Scatter plot showing the mutual changes in gene expression and DNA methylation levels at active enhancers between S3+/+ and S3-/- cells. For each gene, the x axis reports the fold change in gene expression and the y axis the average methylation difference; red dots indicate genes for which both changes reached statistical significance (adjusted *P* value < 0.01 for gene expression, Q-value < 0.01 for methylation difference). See also Supplementary table 5.

d, Volcano plot showing the significant differentially methylated CpG sites between S3+/+ 2i and S3+/+ 2iLIF cells, as described in panel a. Similarly to S3-/- cells, the analysis confirmed the overall gain in DNA methylation levels also in S3+/+ 2i samples, with 512,877 hyper-methylated CpG sites and only 1,506 hypo-methylated sites out of a total of **xxx**sites mapped. Similar effects of LIF were observed also in an independent wild-type mES cell line (E14) (S3c).

e, Scatter plot showing the mutual changes in gene expression (RNAseq) and DNA methylation levels (RRBS) at active promoters between S3+/+ 2i and S3+/+ 2iLIF cells, as described in panel b.

f, Scatter plot showing the mutual changes in gene expression and DNA methylation levels at active enhancers between S3+/+ 2i and S3+/+ 2iLIF cells, as described in panel c.

g, IGV screenshot of RRBS data (in red) and RNAseq data (in blue) for S3+/+ 2iLIF, S3+/+ 2i and S3-/- 2iLIF cells over a representative genomic region. One representative biological replicate of two is reported for RNAseq and RRBS data.

h, Venn diagram showing number of CpG sites whose methylation status is dependent on LIF (light blue circle) or on Dnmt3a/b (red circle). Gray intersection contains the number of CpG sites that lose DNA methylation both when LIF is added to culture medium or when Dnmt3a/b are genetically deleted. Similar results were obtained in an independent Dnmt3a/b dKO clone (Fig. S3e).

i, Scatter plot comparing the effects of adding LIF (y axis) and deleting Dnmt3a/b (x axis) on the levels of CpG methylation in wild-type E14 cells. For each CpG site, the x axis reports the difference in methylation levels (% mCG) between Dnmt3a/b dKO.A and E14 cultivated in 2i, and the y axis the difference in methylation levels between E14 2iLIF and E14 2i. Grey dots indicate all CpG sites that were commonly covered in at least one technical replicate of each sample with a minimum sequencing depth of 10x; blue dots indicate sites that are hypo-methylated both in the presence of LIF or when Dnmt3a/b are genetically deleted (Q-value < 0.01, ΔmCG < -10 %). Similar results were obtained in an independent Dnmt3a/b dKO clone (Fig. S3f).

j, Heatmap reporting percentage of DNA methylation at imprinted Differentially Methylated Regions (DMRs) in S3+/+ and S3-/- cultured in 2iLIF for 22 and 24 passages, respectively. Out of 24 analysed regions, 20 were found significantly more methylated in S3-/- cells compared to S3+/+ cells. n = 2 biological replicates for each sample. See also Supplementary table 5.

k, MeDIP-qPCR analysis of imprinted DMRs in S3+/+ and S3-/- cells. Real-time qPCR was carried out on enriched methylated DNA fractions immunoprecipitated ("IP") from genomic DNA with an antibody specific for 5mC (MeDIP). Negative controls were included, where a non-specific antibody (IgG) was used for immunoprecipitation ("mock"). Enrichment was calculated as % of Input and mean normalised for each experiment. Imprinted DMRs like

Nnat and Peg10 show enrichment in immunoprecipitated fractions, thus higher methylation levels, in S3-/- cells compared to S3+/+ cells; Kif27 was found unchanged in RRBS data, therefore it was included as a control. Bars represent the mean and SD of n = 4 (Nnat, Kif27) or n = 2 (Peg10) independent MeDIP experiments, shown as dots. See also Fig. S3h.

l, Heatmap showing imprinted genes associated with known DMRs (Fig. 2e) and differentially expressed between S3-/- cells and S3+/+ cells in 2iLIF. Expression values were scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. n = 2 biological replicates for each sample. See also Fig. S3i-j.

m, Pie charts showing the number of up- and down-regulated genes (adjusted *P* value < 0.01, abs(log2 FC) > 1) in S3-/- cells with respect to S3+/+ cells among all expressed genes (left), or among all expressed imprinted genes (right).

FIGURE 3

Fig. 3. Stat3 controls DNA methylation via metabolic regulation

a, 5mC immunofluorescence staining on S3+/+ cells cultured in Serum LIF, 2i or 2iLIF, S3-/- cells in 2iLIF and two independent clones, named MitoS3.A and MitoS3.B, where Stat3 is present only in mitochondria (Fig. S4d-h). Violin plots show the distribution of fluorescence intensity of an average of 55 nuclei per sample, normalized to the mean intensity of S3+/+ 2iLIF. Boxplots show 1st, 2nd and 3rd quartile; n = 3 independent experiments are shown as individual violins. Whiskers indicate minimum and maximum values. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the conditions in analysis. Scale bar: 20μm.

b, Mass Spectrometry showing percentages of 5mC in the DNA of S3+/+, S3-/- cells and two MitoS3.A/B clones, cultured in 2iLIF. 5mC content is expressed as the percentage of 5mC in the total pool of cytosines. Bars indicate mean +/- s.e.m. of n = 4 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test.

c, Heatmap reporting differentially expressed imprinted transcripts between S3-/- cells and MitoS3.A/B clones cultured in 2iLIF. Expression values were scaled and represented as z-score. Mean of n = 2 biological replicates is represented for each sample. The genes shown are significantly differentially expressed (adjusted *P* value < 0.1) in both MitoS3 clones relative to S3-/- cells.

d, Histogram showing quantification of individual metabolite abundance measured by Mass Spectrometry; bars indicate mean +/- s.e.m. of n = 5 biological replicates, shown as dots. *P* values were calculated using two-tailed unpaired T-test and are shown only when <0.05.

e, Metabolic tracing analysis of different isotopomers of TCA cycle intermediates (Oxaloacetate, Citrate and αKG) using [U-$^{13}C_5$]-Glutamine. Histogram represents mass isotopomer distribution (MID, the percentage of labelled isotopomer) at 3 different time points (2h, 4h, 8h). Each bar represents mean +/- s.e.m of n = 6 biological replicates. *P* values were calculated using two-tailed unpaired T-test and are shown only when <0.05.

f, Diagram representing mass isotopomer distribution (MID) of Oxaloacetate, Citrate and αKG in both oxidative and reductive Glutamine pathways; MID was analysed following 8h of metabolic tracing with [U-$^{13}C_5$]-Glutamine. Orange box indicates the mitochondrion. Color scale outlines the comparison between MID profile in S3-/- cells with respect to S3+/+ for n =

6 biological replicates, where blue color indicates isotopomers (or biochemical pathways) under-represented is S3-/- cells and red color isotopomers or pathways over-represented in S3-/- cells with respect to S3+/+ cells. Each isotopomer is corrected for natural isotope abundances.

g, Histogram showing quantification of αKG abundance measured by Mass Spectrometry; bars indicate mean +/- s.e.m. of n = 5 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test.

h, Histogram showing quantification by Mass Spectrometry of αKG abundance in S3+/+ 2iLIF cells cultured with Glutamine, without Glutamine for 9 days or without Glutamine but supplemented with 2mM α-ketoglutarate (αKG 2mM) or the cell-permeable dimethyl 2-oxoglutarate (DM-αKG 2mM) for 9 days; bars indicate mean +/- s.e.m. of n = 5 biological replicates, shown as dots. Note that absence of glutamine determines a significant reduction in the levels of αKG, while addition of cell-permeable DM-αKG rescues αKG abundance to levels comparable to S3+/+ 2iLIF with Glutamine. *P* values calculated using two-tailed unpaired T-test.

i, Immunofluorescence staining of 5mC in S3+/+ cells cultured with Glutamine, without Glutamine for 9 days or without Glutamine but supplemented with cell-permeable dimethyl 2-oxoglutarate (DM-αKG 2mM) for 9 days. Left: Violin plots show the distribution of fluorescence intensity of an average of 96 nuclei per sample, normalized to mean intensity of S3+/+ 2iLIF. Boxplots show 1st, 2nd and 3rd quartile; Whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Right: representative images of the 3 conditions in analysis. Scale bar: 20μm.

FIGURE 4

Fig. 4. Alpha-Ketoglutarate regulates 5mC mainly via control of Dnmt3a/b levels

a, Histogram showings the αKG/Fumarate and αKG/Succinate ratios measured by Mass Spectrometry, in S3+/+, S3-/- cells and two MitoS3.A/B clones, cultured in 2iLIF; bars indicate mean +/- s.e.m. of n = 5 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test.

b, Histogram showing h5mC/5mC ration measured by Mass Spectrometry, in the DNA of S3+/+, S3-/- cells and two MitoS3.A/B clones, cultured in 2iLIF; bars indicate mean +/- s.e.m. of n = 5 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test.

c-d, Mitochondrial Stat3 modulates the expression of the *de novo* DNA methyltransferases. Expression analysis of Dnmt3a, Dnmt3b and Tet2 in S3+/+ and S3-/- cells and two MitoS3.A/B clones in 2iLIF by RNAseq (**c**) and RT-qPCR (**d**).
Heatmap (**c**) reports RNAseq expression values scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. n = 2 biological replicates for each sample. Bars (**d**) indicate mean +/- s.e.m. of n = 6 different experiments, shown as dots. Expression values were mean-normalised. Beta-actin serves as an internal control. *P* values calculated using two-tailed unpaired T-test relative to S3-/-.

e, Volcano plot of proteomics data, depicting differences in protein abundances between S3-/- and MitoS3.A cells cultivated in 2iLIF. Each dot represents one protein. The x axis shows the fold change (FC) in protein abundance (measured as LFQ intensity, in log-scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Yellow and blue dots indicate respectively proteins that are less or more (FC > 1 or FC < -1 respectively, adjusted *P* value < 0.05) in S3-/- cells with respect to MitoS3.A cells.

f-g, Gene expression analysis by RT–qPCR of epigenetic modifiers (**f**) and imprinted genes (**g**) in S3+/+, S3-/- and S3-/- cells cultured in 2iLIF and treated with 2mM DM-αKG for 4 passages. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n = 4 independent experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to S3-/-.

h, Gene expression analysis by RT–qPCR of S3+/+ (blue), S3- /- (red) and two MitoS3.A/B (orange) clones cultured with 2iLIF. Data show expression of genes identified as activators (Gold) and repressors (Grey) of Dnmt3a/b. Data are normalized to the mean of n ≥ 3 independent experiments. Beta- actin served as an internal control. Bars indicate mean +/- s.e.m. of n ≥ 3 independent experiments, shown as dots. Two- tailed unpaired T- test relative to S3- /- for each time point.

i, Gene expression analysis by RT–qPCR of S3+/+, S3-/- and S3-/- cells cultured in 2iLIF and treated with 2mM DM-αKG for 3 passages. Data show expression of genes identified as activators (Gold) and repressors (Grey) of Dnmt3a/b. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n ≥ 4 independent experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to S3-/-.

j, Gene expression analysis by RT–qPCR of E14 and Otx2-/- cells stably cultured in 2iLIF or 2i. Data show expressions of Dnmt3a, Dnmt3b and Otx2. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n = 3 independent experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to E14 2i cells.

k, Immunofluorescence on E14 and Otx2-/- cells stably cultured in 2iLIF or 2i stained with anti-5mC antibody. Left: Violin plots show the distribution of fluorescence intensity of an average of 111 nuclei per sample, normalized to the mean intensity of E14 2iLIF. Boxplots show 1st, 2nd and 3rd quartile; Whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Right: representative images of the 4 conditions in analysis. Scale bar: 20μm.

FIGURE 5

Fig. 5. Mitochondrial Stat3 regulates differentiation of ES cells.

a, Volcano plot showing differentially expressed genes between S3+/+ and S3-/- cells. Each dot represents one gene. The x axis shows the fold change (FC) in expression levels (in log-scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Yellow and blue dots indicate respectively transcripts that are up-regulated or down-regulated (log2 FC > 1 or log2 FC < -1 respectively, adjusted *P* value < 0.01) in S3-/- cells with respect to S3+/+ cells.

b, Boxplot reporting expression levels of down-regulated genes in S3-/- cells with respect to S3+/+ cells (Fig. 5a, blue dots). Each boxplot shows 1st, 2nd and 3rd quartile. Whiskers show minimum and maximum values. Y axis represents mean-normalized TPM (Transcripts Per Million) for S3+/+, S3-/- and Mito-Stat3 clones (MitoS3.A and MitoS3.B) in two different conditions: following stable culturing of cells in 2iLIF (light blue) and after 48h of 2iLIF withdrawal from culture medium (dark blue). Upper table shows mean log2 FC in gene expression for all analysed conditions, each compared to S3+/+ 2iLIF.

c, Boxplot reporting expression levels of up-regulated genes in S3-/- cells with respect to S3+/+ cells (Fig. 5a, yellow dots). Y axis represents mean-normalized TPM for S3+/+, S3-/- and Mito-Stat3 clones (MitoS3.A and MitoS3.B) in two different conditions: following stable culturing of cells in 2iLIF (light yellow) and after 48h of 2iLIF withdrawal from culture medium (dark yellow). Clones expressing only mitochondrial Stat3 (MitoS3.A and MitoS3.B) show a substantial rescue of gene expression levels compared to S3-/- cells, both in 2iLIF and after 48h of LIF withdrawal. Upper table shows mean log2 FC in gene expression for all analysed conditions, each compared to S3 +/+ 2iLIF.

d, Heatmap for 8 pluripotency-associated markers in mES cells. RNAseq data derived from S3+/+, S3-/-, MitoS3.A and MitoS3.B expanded in 2iLIF or without 2iLIF for 48h. Expression levels were scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. Note that S3-/- cells display reduced levels of naive markers compared to S3+/+ cells. n = 2 biological replicates for each sample.

e, Heatmap for 10 imprinted genes (upper part) and 6 early differentiation markers (lower part) in mouse ES cells. RNAseq data derived from S3+/+ and S3-/- cells and two independent clones expressing Stat3 localized only in the mitochondria (MitoS3.A and MitoS3.B) expanded in 2iLIF or without 2iLIF for 48h. Expression values were scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. Note that S3-/- cells display much faster upregulation of early differentiation markers and imprinted genes after 48 hours in N2B27; moreover, mitochondrial Stat3 clones show a rescue in the expression of these transcripts compared to S3-/- cells. n = 2 biological replicates for each sample.

f, Gene expression analysis by RT–qPCR of S3+/+ (blue), S3-/- (red) and two MitoS3.A/B (orange) clones cultured with 2iLIF or without 2iLIF for 24h or 48h. Early differentiation markers are more readily induced in S3-/- and MitoS3.A/B clones rescues this effect. Data are normalized to the mean of n = 3 independent experiments. Beta-actin served as an internal control. Bars indicate mean and s.e.m. of n = 3 independent experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to S3-/-. See also Fig. S4.

g, Alkaline phosphatase (AP) staining in S3+/+, S3-/- and MitoS3.A/B clones cultured with 2iLIF or without 2iLIF for 24h, 48h or 72h. Right: Number of AP-positive colonies, relative to S3+/+ cells in 2iLIF. Mean +/- s.e.m. of n = 3 independent experiments is shown.

FIGURE 6

Fig. 6. Stat3 regulates Dnmts and imprinted transcripts in the early mouse blastocysts.

a, Schematic outline of the single-cell isolation from embryos and profiling. A total of 171 cells from 18 embryos were analysed (See Supplementary table 3).

b, t-Distributed Stochastic Neighbor Embedding (t-SNE) based on whole transcriptome of wild-type (S3+/+) and mutant (S3-/-) cells collected at embryonic day E3.5; each dot represents a single cell.

c, Volcano plot of genes differentially expressed between S3-/- and S3+/+ cells at E3.5. Each dot represents one gene. The x axis shows the fold change in expression levels (in log-scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Red and blue dots indicate respectively transcripts that are upregulated or downregulated (log2 FC > 0.7 or log2 FC < -0.7 respectively, adjusted *P* value < 0.1) in S3-/- cells relative to S3+/+ cells. See also Supplementary table 6.

d, Violin plots showing the distribution of expression levels (log2 FPKM+1) for selected markers in S3+/+ (blue) and S3-/- (red) cells collected at E3.5. Boxplots show 1st, 2nd and 3rd quartile. Whiskers indicate minimum and maximum values. Note that Stat3 and its target Tfcp2l1 are downregulated in S3-/- cells at E3.5 while post implantation epiblast markers (i.e. Dnmt3a, Dnmt3b) and PrE markers (i.e. Sox17, Pdgfra) are upregulated in S3-/-. * = *P* value <0.05, two-tailed unpaired T-test.

e, t-SNE based on genome-wide expression of S3+/+ and S3-/- mouse cells collected at embryonic day E3.75; each dot represents a single cell. Clustering resolves distinct sample

groups by embryonic cells type (Epiblast - Epi and Primitive Endoderm - PrE) along the first dimension, and by genotype (S3+/+ and S3-/-) along the second dimension.

f, Volcano plot of genes differentially expressed between S3-/- and S3+/+ cells at E3.75. Each dot represents one gene. The x axis shows the fold change in expression levels (in log-scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Red and blue dots indicate respectively transcripts that are upregulated or downregulated (log2 FC > 0.7 or log2 FC < - 0.7 respectively, adjusted *P* value < 0.1) in S3-/- cells with respect to S3+/+ cells. See also Supplementary table 6.

g, Violin plots showing the distribution of gene expression levels (log2 FPKM+1) for S3+/+ (blue) and S3-/- (red) ES cells collected at E3.75 . Boxplots show 1st, 2nd and 3rd quartile. Whiskers indicate minimum and maximum values. * = *P* value <0.05, two-tailed unpaired T-test.

h, Heatmap reporting average expression levels of imprinted transcripts in three different embryonic populations (E3.5 ICM, E3.75 Epi, E3.5 PrE) from S3+/+ and S3-/- embryos. Expression values were scaled and represented as z-score. Yellow and blue indicate high and low expression, respectively. Only expressed imprinted genes (average FPKM >1) we analysed.

i, Violin plots showing the distribution of expression levels (log2 FPKM+1) of imprinted genes for S3+/+ (blue) and S3-/- (red) ES cells collected at E3.5, 3.75 Epi and 3.75 PrE. Boxplots show 1st, 2nd and 3rd quartile. Whiskers indicate minimum and maximum values. * = *P* value <0.05, two-tailed unpaired T-test.

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Author contributions:

G.M., N.M. and S.O. designed the study; R.M.B. and L.D. performed ES cell culture, molecular characterization and functional assays and visualization; V.P. and L.D. performed MeDIP qPCR; R.M.B., V.P. and S.R. performed RRBS; R.M.B. and S.R. performed Western Blots; A.L. and D.I. performed RRBS integrated analysis; M.A. and L.D. performed RNAseq analysis; R.M.B., S.P. and M.A. performed metabolomic analyses; R.M.B., V.P., V.G. and D.T. performed nucleotide Mass Spectrometry; L.D. and G.G.S. performed single-cell RNAseq analysis; T.L., T.B. and J.N performed embryo dissection and single-cell RNAseq library preparation; G.M. wrote the manuscript with inputs from all authors; G.M., N.M. and S.O. supervised the study.

Competing interests:

The authors declare no competing interests.

Data and materials availability:

Bulk and single-cell RNA sequencing data and RRBS data generated during the current study are available via the Gene Expression Omnibus (GEO) repository under the accession numbers GSE133926 and GSE134450. All RNA-sequencing and RRBS process data, used in Figures 1d, 2a-c, 2g-h, 3g, 4b-g, 5a-e, 6b-i, S2e, S5 are reported in Supplementary tables 4, 5 and 6 and via the Gene Expression Omnibus (GEO) repository under the accession numbers GSE133926. RNA sequencing data of Rex1-GFPd2 cells can be found at GEO under accession number GSE111694. Mass spectrometry proteomics data of following samples: S3+/+ cells in 2i; S3+/+, S3-/- , MitoS3.A and MitoS3.B cells in 2iLIF; used in Figures 1f, 4e , S1c are reported in Supplementary tables 7 and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020385. Additional data that support the findings of this study, such as Mass Spectrometry measurements, and reagents are available from the corresponding authors upon reasonable request.

Methods

Cell lines and culture routine

Wild type or Stat3 KO (described previously in [13,62] and provided by A. Smith's Lab) mouse ES cell lines were routinely cultured without feeders on gelatine-coated plates (0.2% gelatine, Sigma-Aldrich, cat. G1890). Media was changed every 2 days and cells were passaged when approaching confluency (every 2-3 days); to passage, cells were replated at required density following dissociation with either Accutase (Thermo-Fisher, cat. A11105-01) or Trypsin (Thermo-Fisher, cat. 15090-046).

All cells were maintained at 37°C in humidified incubators with 5% CO2.

Media and supplements

Cells were grown under two different culture conditions, prepared as follows:

2i / 2iLIF: serum-free KSR (Knockout serum replacement) 10% (Life Technologies, cat. 10828-028) - based medium in GMEM (Sigma-Aldrich, cat. G5154) supplemented with 1% FBS (Sigma-Aldrich, cat. F7524), 100 mM 2- mercaptoethanol (Sigma-Aldrich, cat. M7522), 1×MEM non-essential amino acids (Invitrogen, cat. 1140-036), 2mM L-Glutamine, 1mM sodium Pyruvate (both from Invitrogen), and 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).

Serum LIF: GMEM (Sigma-Aldrich, cat. G5154) supplemented with 10% FBS (Sigma-Aldrich, cat. F7524), 100 mM 2-mercaptoethanol (Sigma-Aldrich, cat. M7522), 1× MEM non-essential amino acids (Invitrogen, cat. 1140-036), 2mM L-Glutamine, 1mM Sodium Pyruvate (both from Invitrogen), and 100 units/ml LIF.

Generation of mutant cell lines

Dnmt3b -/- ESCs Knockout production was performed using TALEN technology as described in Neri et al. [73]. In brief, cells were transfected with the two TALEN constructs targeting Exon 17 of murine Dnmt3b and after 16 hours were seeded as a single cell. After 1 week, clones were screened by western blot analyses. Positive clones were analyzed by genomic sequencing of the TALEN target.

Dnmt3a-/- and Dnmt3ab-/- ESCs generation were performed by CRISP-R/ Cas9 method. E14 Wild-type cells and Dnmt3b-/- were co-transfected with Cas9 construct and the two RNA guide construct targeting Exon 19 (FW gRNA exn19= CACCgACCGCCTCCTGCATGATGCGCGG, REV gRNA exn19= aaacCCGCGCATCATGCAGGAGGCGGTc) of murine Dnmt3a. After 16 hours single cells sorting was carried out. Ten days later, clones were screened by western blot analysis. Positive clones were analysed by genomic sequencing.

For DNA transfection, we used Lipofectamine 2000 (Life Technologies, cat. 11668-019) and performed reverse transfection. For one well of a 12-well plate, we used 3 ul of transfection reagent, 1 ug of plasmid DNA, and 150,000 cells in 1 ml of KSR medium. The medium was changed after overnight incubation.

Stable transgenic ESCs lines expressing sh-TET1/2, sh-Scramble or MLS-Stat3-NES were generated by transfecting cells with PiggyBac transposon plasmids CAG-sh-TET1/2, CAG-sh-Scramble or CAG-MLS-Stat3-NES with piggyBac transposase expression vector pBase. Selection for transgenes was applied, and stable clones were selected in 2iLIF conditions.

STAT3ER plasmid transfection previously described in Takahiko Matsuda et al. 1999 [74] was performed with a 1ug of linearized plasmid (enzyme PbuI). Plasmid enconde for the entire coding region of mouse STAT3 followed by the modified ligand binding domain (G525R) of mouse estrogen receptor under control of CAG promoter (pCAGGS vector).

Dnmt3a1/2 costructs for Overexpression experiments was obtained by PCR amplification of entire coding region (Dnmt3a1 or Dnmt3a2) and cloned into the XbaI–NotI site of pEF6/V5-His vector (Invitrogen). Dnmt3b construct was obtained by PCR amplification and cloned into pEF6/V5-His vector (Invitrogen) previously described in Neri et al. 2017 Nature[73].

LIF induction

For LIF induction experiments, ES cells cultured in 2i without LIF for at least 4 passages were plated in 2i. Twenty-four hours after plating, LIF was added for the indicated amount of time (24 and 48 hours), cells were then fixed for immunofluorescence.

Treatments

For inhibition of the respiratory chain, cells were treated acutely with 100nM complex I inhibitor Rotenone (Sigma-Aldrich, cat. R8875) and 200nM complex III inhibitor Antimycin (Sigma-Aldrich, cat. A8674).

For studies on glutaminolysis, cells were cultured in KSR-based medium prepared as described above, but without the addition of Glutamine. Exogenous DM-αKG (dimethyl 2-oxoglutarate) used for treatments was added to culture medium at the indicated concentration and absorbed by cells as it is membrane-permeable (Sigma-Aldrich, cat. 349631-5G).

Clonal assay

The ability of single mES cells to form pluripotent colonies was assessed through a clonal assay. Cells were harvested by trypsinization and plated at clonal density; to do this, they were counted and diluted to obtain a final number of 600 cells/well. Cells were grown for 4-5 days before they were fixed and stained for Alkaline Phosphatase.

Differentiation assay

Cells were cultured with or without 2iLIF for 24, 48 and 72 hours. After 72 hours cells were detached and replated at clonal density in 2iLIF. Cells surplus were conserved for gene expression analysis. Finally, cells were stained with Alkaline Phosphatase after 5 days to evaluate the number of pluripotent cells. For AP staining, cells were fixed with a citrate-acetone-formaldehyde solution and stained using the Alkaline Phosphatase kit (Sigma-Aldrich, cat. 86R-1KT). Plates were scanned using a Nikon Scanner and scored manually.

Alkaline Phosphatase Staining

Fixation solution: 65% Acetone, 25% Citrate (provided with kit), 8% Formaldehyde

Staining solution: Alkaline Phosphatase (AP) kit (Sigma-Aldrich, cat. 86R-1KT) according to manufacturer's protocol.

Culture medium was removed from adherent cells and they were fixed with fixation solution. Plates were then washed with H2O and staining solution was added for 5 minutes in the dark. Then plates were washed again with H2O and dried.

Colonies were scored manually using optical microscope, discriminating between undifferentiated (AP-positive), mixed or differentiated (AP-negative).

Immunofluorescence and stainings

For 5mC staining, cells were fixed in 4% formaldehyde (Sigma-Aldrich, cat. F8775) for 10 minutes, then washed in PBS and treated for 15 minutes with NH4Cl. Next, cells were permeabilized with 1h PBST 0.5% treatment (PBS, 0.5% Triton X-100, Sigma-Aldrich, cat. 93443) and 2N HCl was added for 45 minutes to denature the DNA. Cells were blocked for 1 hour in 5% horse serum (HS) with 0.3% PBST (Thermo-Fisher, cat. 16060122) and then incubated overnight at 4°C with anti-5mC primary antibody (Eurogentec, cat. BI-MECY-0500, Supplementary table 1) diluted in 2% HS with 0.3% PBST. After washing with PBST 0.1%, cells were incubated with secondary antibody (Alexa Fluor 488 donkey anti-mouse, Life Technologies, cat. A21202) for 45 minutes at room temperature (RT). Nuclei were stained with mounting medium Fluoroshied containing DAPI (Sigma-Aldrich, cat. F6057).
For EdU staining, cells were exposed to an EdU pulse of 4 hours before fixation in 4% formaldehyde for 10 minutes; samples were then processed according to manufacturer's instructions (Life Technologies).
For Atad3 and Stat3 colocalization staining, cells were fixed for 10 minutes in 4% formaldehyde, washed in PBS and blocked and permeabilised for 1 hour in 5% horse serum (HS) with 0.3% PBST. Cells were incubated overnight at 4°C with primary antibodies (Supplementary table 1). After washing with 0.1% PBST, cells were incubated with secondary antibodies (Alexa, Life Technologies) for 30 minutes at RT. Nuclei were stained with mounting medium Fluoroshied containing DAPI.
Images were acquired with a Leica SP5 or a Zeiss LSM 700 confocal microscope equipped with a CCD camera. Fluorescence intensity was quantified using the freely available software Fiji (http://fiji.sc/Fiji).

Oxygen consumption assay (Seahorse Assay)

Oxygen consumption was measured using the Seahorse XF24 (Seahorse Bioscience). For this, 20 hours before the analysis both S3+/+ and S3-/- cells were seeded in a 24-well cell culture plate (Seahorse Bioscience) coated with laminin (Sigma-Aldrich, cat. L2020) at a density of 100,000 cells per well in KSR media supplemented with 2i + LIF. It is crucial to have an evenly plated mono- layer of cells to obtain reliable measurements. Cells were maintained in a 5% CO2 incubator at 37°C, and 1 hours before the experiment, the cells were washed and incubated in 600 µl of DMEM (Sigma-Aldrich, cat. D5030-10X1L) with 2mM Glutamine, 1mM NaPy, 25 mM glucose, 3 mg/L phenol red and 143 mM NaCl, with 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 minutes intervals, consisting of a 3-min mixing period and 2 minutes waiting period. Oxygen consumption rate (OCR) is measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration. Initially, cellular OCR is measured in basal conditions to derive the basal mitochondrial respiration; next, 200 nM mitochondrial uncoupler FCCP (carbonyl cyanide-p- trifluoromethoxyphenyl-hydrazon) is automatically added to the medium to maximize Electron Transport Chain (ETC) function, in order to derive maximal respiratory capacity. Next, Antimycin A and Rotenone - inhibitors of complex III and I - are released into the medium to block ETC, revealing the non-mitochondrial respiration.

Gene expression analysis by quantitative PCR with reverse transcription

Total RNA was isolated using a Total RNA Purification kit (Norgen Biotek, cat. 37500), and complementary DNA (cDNA) was made from 500 ng using M-MLV Reverse Transcriptase (Invitrogen, cat. 28025-013) and dN6 primers (Invitrogen). For real-time PCR, SYBR Green Master mix (Bioline, cat. BIO-94020) was used. Three technical replicates were carried out

for all quantitative PCR. An endogenous control (beta-actin) was used to normalize expression. Primers are detailed in Supplementary table 2.

RNAseq

Total RNA was isolated using Total RNA Purification kit (Norgen Biotek, cat. 37500) and sequenced using an Illumina HiSeq4000, in 150-base pair paired end format.

Reads were aligned to mouse transcriptome (Mus musculus transcriptome generated by rsem-prepare-reference with ENSEMBL93 GTF) and mouse genome (GRCm38.p6) using HISAT2 v. 2.1.0.

Gene expression levels were quantified with RSEM v. 1.3.1 using transcriptome alignments. Genome alignment were used to create bigWig files using deeptools (v. 3.2.1).

Genes were sorted based on average expression calculated in a total of 18 samples, and final expression matrix was generated excluding genes that had an average expression lower than 22.88 raw counts; after applying this filter, we obtained expression of 12,510 genes.

All RNAseq analyses were carried out in R environment (v. 3.5.3) with Bioconductor (v. 3.7)

We computed differential expression analysis using the DESeq2 R package (v. 1.24.0, Love *et al.*, 2014); DESeq2 performs the estimation of size factors, the estimation of dispersion for each gene, and fits a generalized linear model. Transcripts with absolute value of log2-fold change > 1 and with an adjusted *P* value (P_{adj}) < 0.01 (Benjamini-Hochberg adjustment) were considered significant and defined as differentially expressed (Differentially Expressed Genes = DEG) for the comparison in analysis.

Heatmaps were made using TPM values with the pheatmap function from pheatmap R package (v.1.0.12, distance = 'correlation', scale = 'row') on DEGs or selected markers.

Volcano plots were computed with $log_2 fold\ change$ and $-log_{10}P_{adj}$ from DESeq2 differential analysis output using ggscatter function from ggpubr R package (v. 0.2).

Western blot

Cells were washed in PBS and harvested with lysis F-buffer (10 mM TrisHCl pH7, 50 mM NaCl, 30 mM Sodium pyrophosphate tetrabasic, 50 mM NaF, 1% Triton X-100 Buffer). In order to obtain protein lysates, extracts were exposed to ultrasound in a sonicator (Diagenode Bioruptor) for 3 pulses. Cellular extracts were centrifuged for 10 minutes at 4°C (max speed) to remove the insoluble fraction. Extracts were quantified using bicinchoninic acid (BCA) assay (BCA protein assay kit; catalog no. 23225; Pierce). Samples were boiled at 95°C for 5 minutes in 1X Sample Buffer (50mM Tris HCl pH 6.8, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol, 2% 2-mercaptoethanol).

Each sample was loaded in a commercial 4-12% MOPS acrylamide gel (Life Technologies; BG04125BOX/BG00105BOX) and electrophoretically transferred on a PVDF membrane (Millipore; IPFL00010) in a Transfer solution (50mM Tris, 40mM glycine, 20% methanol, 0.04% SDS). Membranes were then saturated with 5% Non-Fat Dry Milk powder (BioRad; 170-6405- MSDS) in TBST (8g NaCl, 2.4g Tris, 0.1% Tween20/liter, pH 7.5) for 1 hour at room temperature and incubated overnight at 4 °C with the primary antibody (Supplementary table 1) diluted in a range of 0,5-1% milk powder (depending on antibody) in TBST. Membranes were then incubated with secondary antibodies conjugated with a peroxidase, diluted in 0,1% or 0,5% milk in TBST. Pico SuperSignal West chemiluminescent reagent (Thermo Scientific, cat. 34078) was used to incubate membranes and chemiluminescence from the interaction between peroxidase and substrate present in the commercial reagent was digitally acquired by ImageQuant LAS 4000.

Metabolites analysis by Mass Spectrometry

Cells were grown in 6-well plates, harvested in ice-cold PBS and centrifuged at 500g for 3 minutes at 4°C. Pellets were then resuspended in 250µl methanol/acetonitrile 1:1 containing [U-$^{13}C_6$]-Glucose-1ng/µl (internal standard, Sigma-Aldrich, cat. 389374) and centrifuged at 20,000g for 5 minutes at 4°C. Supernatant were then passed through a regenerated cellulose filter, dried and resuspended in 100µl of MeOH for subsequent analysis.

Metabolomic data were performed on an API-4000 triple quadrupole Mass Spectrometer (Sciex) coupled with a HPLC system (Agilent) and CTC PAL HTS autosampler (PAL System) and on API3500 (Sciex)The identity of all metabolites was confirmed using pure standards.

Quantification of different metabolites was performed with a liquid chromatography/tandem Mass Spectrometry (LC-MS/MS) method using a C18 column (Biocrates) for amino acids and SAMe and cyano-phase LUNA column (50mm x 4.6mm, 5µm; Phenomenex). Methanolic samples were analyzed by a 10 minutes run in positive (amino acids and SAME) and 5 minutes run in negative (all other metabolites) ion mode with specific multiple reaction monitoring (MRM) transitions. Amino acids quantification was performed through previous derivatization. Briefly, 50µl of 5% phenyl isothiocyanate (PITC) in 31.5% EtOH and 31.5% pyridine in water were added to 10µl of each sample. Mixtures were then incubated with PITC solution for 20 min at RT, dried under N2 flow and suspended in 100µl of 5mM ammonium acetate in MeOH/H2O 1:1. The mobile phases for positive ion mode analysis (amino acids and SAMe) were phase A: 0.2% formic acid in water and phase B: 0.2% formic acid in acetonitrile. The mobile phase for negative ion mode analysis (all other metabolites) was phase A: water and B: 2 mM ammonium acetate in MeOH. The gradient was 90% B for all the analysis with a flow rate of 500ul/min. MultiQuantTM software (version 3.0.2) was used for data analysis and peak review of chromatograms. Quantitative evaluation of all

metabolites was performed based on calibration curves with pure standards, then data were normalized on total protein content.

Metabolic flux analysis

For metabolic tracing analyses, cells were exposed for 24h to [U-$^{13}C_6$]-Glucose 1mM (Sigma-Aldrich, 389374) or [U-$^{13}C_5$]-Glutamine 2mM (Sigma-Aldrich, cat. 605166) or [U-$^{13}C_{16}$]-Palmitate 100µM (Sigma-Aldrich, cat. 605573). Cells were harvested in ice-cold PBS and centrifuged at 500g for 3 minutes at 4°C. Pellets were then resuspended in 250µl methanol/acetonitrile 1:1 and spun at 20,000g for 5 min at 4°C. Supernatant were then passed through a regenerated cellulose filter, dried under N2 flow and resuspended in 100µl of MeOH for subsequent analysis. Metabolomic data were performed on an API-4000 triple quadrupole Mass Spectrometer (Sciex) coupled with a HPLC system (Agilent) and CTC-PAL HTS autosampler (PAL System). The identity of all metabolites was confirmed using pure standards. Quantification of different metabolites was performed with a liquid chromatography/tandem Mass Spectrometry (LC-MS/MS) method using a cyano-phase LUNA column (50mm x 4.6mm, 5µm; Phenomenex) and corrected for natural isotope abundances. Methanolic samples were analyzed by a 5 min run in negative (Metabolites) ion mode. The mobile phases for negative ion mode analysis was phase A: 2 mM ammonium acetate in MeOH and phase B: water. The gradient was 90%A for all the analysis with a flow rate of 500µl/min. MultiQuantTM software (version 3.0.2) was used for data analysis and peak review of chromatograms. Samples were analysed after 8 hours of 13C-labelling to ensure that isotopic equilibrium was reached, as previously shown in ES cells cultured in 2iLIF by Carrey and colleagues.

Nucleoside preparation for Mass Spectrometry

DNA was extracted using Puregene core kit A, then measured with a Nanodrop spectrophotometer. 50µg DNA were passed through the Microcon YM-10 centrifugal filtration cartridge (Millipore, cat. no. 42407, MRCPRT010) 10KDa columns two times. The first time 50µg of DNA were solubilized into 500µL of double distilled water, then concentrated to about 30 µL by spinning the columns at 13900g for 25 minutes. The second time, the 30µL of recovered DNA were solubilized into 500µL of 1X digestion buffer and then concentrated to about 15µL by spinning at 13900g for 35 minutes.
After the 2 steps, the DNA concentration was measured at the Nanodrop spectrophotometer. The DNA was then digested to nucleosides, at 37°C for 6-7 hours, using a mix containing 2U Antarctic Phosphatase (stock solution is 5U/µl) (New England Biolabs, M0289S), 3mU Snake venom phosphodiesterase I (stock solution is 1mU/µl) (Crotalus adamanteus venom, Sigma-Aldrich, P3243-1VL), 2.5U Benzonase (stock solution is 250U/µl) (Sigma-Aldrich E1014-5KU), in 3.4µl volume of enzyme mix + 1.6µl of double distilled water + 5µl 2X digestion buffer (20mM Tris Hcl pH 7.9 100mM NaCl, 20mM MgCl2) + 5µl of DNA (7,5 – 10µg) in 1X digestion buffer. After the digestion 1µg of undigested genomic DNA and 1µg of digested DNA were loaded on a 2% gel, in order to confirm the complete digestion of the genomic DNA. 12µl of digested nucleosides were provided for MS analysis to CNRS at Gif-sur-Yvette [75].

Mass Spectrometric Analysis of Total Nucleosides

Analysis of the nucleoside digests of DNA by HPLC was performed with a U-3000 HPLC system (Thermo-Fisher). An Accucore RP-MS (2.1 mm X 100 mm, 2.6 µm particle, Thermo-Fisher) column was used at a flow rate of 200 µL/min and a fixed temperature at 30°C. Mobile phases were 5 mM ammonium acetate, pH 5.3 (buffer A) and 40% aqueous

acetonitrile (Buffer B). A multilinear gradient was used with only minor modifications from that described previously [76]. The injection volume was fixed at 6 µL.

A LTQ orbitrap Mass Spectrometer (Thermo-Fisher) equipped with an electrospray ion source was used for the HPLC-MS identification and quantification of nucleosides. Mass Spectra were recorded in the positive ion mode over an *m/z* range of 100-1000 with a capillary temperature set at 300°C, spray voltage at 4.5 kV and sheath gas, auxiliary gas and sweep gas at 40, 12 and 7 arbitrary units, respectively.

Calibration curves were generated using a mixture of synthetic standards of 2'-Deoxycytidine (2dC)(Sigma-Aldrich), 5-Methyl-2'-deoxycytidine(5-mdC) and 5-hydroxymethyl-2'-deoxycytidine (5-hmdC) (Bertin-Pharma) in the ranges of 10-100 injected pmol for 2dC, 0.4-4 injected pmol for 5-mdC and 0.5-10 injected pmol for 5-hmdC. Each calibration point was injected in triplicate. Extracted Ion Chromatograms (EIC) of base peaks of the following signals: 2dC (*m/z* 228.08-228.12), 5-mdC (*m/z* 242.10-242.13), and 5-hmdC (*m/z* 258.08-258.12), were used for quantification. In all cases, coefficients of variations for peak areas were always below 15%. Experimental data (peak area *versus* injected quantity) were fitted with a linear regression model for each compound leading to coefficient of determination (R^2) values better than 0.97. Accuracies were calculated for each calibration point and were always better than 15%.

MEDIP

Genomic DNA was extracted with phenol chloroform, resuspended in TE buffer containing 20µg/ml RNAse A (Thermo-Fisher, cat. EN0531) and passed through a needle 10 times to reduce its viscosity, then measured at the Nanodrop spectrophotometer.

40µg of DNA were resuspended in 130µl of TE, transferred to a microtube (microtubes AFA fiber pre-slit snap cap 6x16mm, Covaris) and sonicated with the Covaris S2 (Duty cycle 10%,

Intensity 5, Cycles burst 200, 45 seconds per cycle; 3 cycles to have a distribution of size between 100 and 600, 4 cycles to have a distribution of size between 100 and 400 and 5 cycles to have a distribution of size between 100 and 300).

10µg of sonicated DNA were diluted in 1,125ml TE and denatured for 10 minutes at 100°C in a thermoblock, then quickly cooled on ice for additional 10 minutes. 450µl (= 4µg tot) of denatured DNA were distributed in two low binding tubes with 51µl of 10X IP buffer (100 mM Na-Phosphate pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100), plus 10µl of antibody anti-5mC (Supplementary table 1) (IP sample) or IgG (mock control) were added. The tubes were left rotating with overhead shaking for 2 hours at 4°C. The leftover (= 225µl) is the Input material (50% of Input), to be used in the quantitative PCR.

Dynabeads Protein G (Thermo-Fisher, cat. 10003D) were prepared by taking 40µl per each sample, then washed twice for 5 minutes in 800µl of 0.1% BSA in PBS and finally resuspended in 40µl of 1X IP buffer.

After 2 hours, Dynabeads Protein G were added to the IP and mock samples. Samples were left rotating at 4°C with overhead shaking for additional 2 hours.

The beads were then separated using the magnetic stand and washed 3 times for 10 minutes in 1X IP buffer; the supernatant was removed and trashed (unbound fraction). Finally the beads were resuspended in 250µl of proteinase K digestion buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5 % SDS) containing 3.5µl of proteinase K (20mg/µl). The samples were incubated overnight at 50°C in a shaking thermoblock (500rpm). The day after the beads were separated with a magnetic rack and the supernatant was saved.

The DNA contained in the supernatant fraction was purified using Qiaquick PCR Purification kit (Qiagen, cat. 2816) and eluted in 30 µl. The saved Input material (50% Input) was re-purified and concentrated using Qiaquick kit; elution was done in a volume of 30µl. Primers are detailed in Supplementary table 3.

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS was performed as previously described in [77]. Briefly, 500ng of DNA was digested at 37°C with 200U of MspI restriction endonuclease (NEB). Digested DNA was than end repaired, dA-tailed, and ligated to methylated adapters, using the Illumina TruSeq DNA Sample Prep Kit, following manufacturer's instructions. Adapter-ligated DNA was loaded on 2% agarose gel and a fraction from 200 to 400 bp was recovered. Purified DNA was then subjected to bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen). Bisulfite-converted DNA was finally enriched by 15 cycles of PCR using Kapa HiFi HotStart Uracil (Roche).

RRBS data processing and analysis

After quality controls, sequencing reads were mapped to mouse genome reference (mm10/GRC.m38.p6) with BSMAP (v2.89) [1] using RRBS mode (parameters: -s 12 -D C-CGG -v 0.01 –n 1). CpG methylation levels were extracted from aligned reads as the ratio of the number of Cs over the total number of Cs and Ts using the methratio.py script. CpG methylation ratios from both strands were combined (parameters: –g). For downstream analysis, the CpG sites that were commonly covered in at least one technical replicate of each sample with a minimum sequencing depth of 10x were retained. All samples were processed identically.

Statistical analyses were conducted within the R software environment. Differential methylation analysis at single nucleotide resolution was performed for each comparison (i.e. Stat3+/+ 2iLIF vs Stat3 -/- 2iLIF; Stat3+/+ 2i vs Stat3 +/+ 2iLIF; Dnmt3a/b dKO.A and dKO.B 2i vs E14 2i; 2iLIF vs 2i E14) using the methylKit R/Bioconductor package [2], exploiting the logistic regression approach for testing replicates (calculateDiffMeth function with default parameters). CpG sites with absolute methylation difference >= 10% and q-value

<= 0.05 were considered as differentially methylated. Correlation analysis between the effect of LIF and Dnmt3a/b on CpG methylation was performed on the methylation difference of each condition with respect to 2i-cultured wild type cells using the cor.test R function.

For the study of DNA methylation levels on regulatory elements, ChIP-seq data of histone marks (H3K27ac and H3K4me3) generated in E14 mES cells were retrieved from ENCODE (https://www.encodeproject.org/). Active promoters and enhancers were defined from processed peaks data as following:

Promoters: H3K4me3 peaks in a 2kb window centred in the TSS of annotated genes (GENCODE release M20);

Enhancers: distal H3K27ac peaks (more than 1kb up/downstream the nearest TSS).

Differential methylation analysis (calculateDiffMeth function, q-value <=0.05, methylation difference >= 10%) was performed on these regions (i.e., testing all the covered CpG overlapping with the ChIP-seq peaks, with 200bp of flanking region) for the comparisons: Stat3+/+ 2iLIF vs Stat3 -/- 2iLIF and Stat3+/+ 2i vs Stat3 +/+ 2iLIF. These results were then integrated with RNA-seq data. After performing differential expression analysis, the fold change in gene expression levels was visualized against the average changes in DNA methylation levels of promoters/enhancers. Similar analyses were conducted on a manually curated list of imprinted DMRs.

Single-cell RNAseq analysis of Stat3-/- and +/+ embryos

Immunosurgery and single-cell dissociation was performed as described in Boroviak 2015 Dev Cell [7]. The method for single-cell RNA-seq and Library preparation was previously described in Boroviak 2018 Dev [78]. A total of 171 cells from 18 embryos were analysed. Experiments were performed in accordance with EU guidelines for the care and use of laboratory animals, and under authority of UK governmental legislation. Use of animals in

this project was approved by the ethical review committee for the University of Cambridge, and relevant Home Office licenses are in place.

Mus musculus GRCm38.87 gene annotation and mm10 genome sequence were downloaded from Ensembl (https://www.ensembl.org/index.html). All reads were aligned using Spliced Transcripts Alignment to a Reference [78]. Alignments to gene loci were quantified with htseq-count [79] based on annotation from Ensembl 87. PCA outliers were computed and removed. Mouse embryo for E4.5, E5.5 and E6.5 stages were compiled from earlier studies[10,80]. Principal component analysis was based on Log2 FPKM values computed with the Bioconductor package DESeq [81], custom scripts and FactoRmineR package [82]. Differential expression analysis was performed with scde [83], that fits individual error models for assessment of differential expression between groups of cells. Fractional identity between E3.5/E3.75 Stat3 +/+ and Stat -/- cells and embryo stages (E4.5 EPI, E5.5 EPI and E6.5 EPI) was computed using R package DeconRNASeq [84] which makes use of quadratic programming to estimate the proportion of distinctive types of tissue. The average expression of the embryo stages was used as "signature" dataset. See also Supplementary table 6.

Proteomics

All the experiments have been performed in a labeling free setting. For each sample, 50mg of total cellular protein extract were precipitate over-night at 4°C in acetone, then reduced and alkylated in a solution of 6M Guanidine-HCl, 5mM TCEP, and 55mM chloroacetamide. Peptides were obtained digesting proteins with LysC (WAKO) for 3h at 37°C and with the endopeptidase sequencing-grade Trypsin (Promega) overnight at 37°C. Generated peptides were extracted using an increasing acetonitrile concentration. Collected peptide mixtures were concentrated and desalted using the Stop and Go Extraction (STAGE) technique (Rappsilber et al., 2003).

Instruments for LC MS/MS analysis consisted of a NanoLC 1200 coupled via a nano-electrospray ionization source to the quadrupole-based Q Exactive HF benchtop mass

spectrometer (Michalski et al., 2011). Peptide separation was carried out according to their hydrophobicity on a PicoFrit column, 75mm ID, 8Um tip, 250mm bed packed with Reprosil-PUR, C18-AQ, 1.9mm particle size, 120 Angstrom pore size (New Objective, Inc., cat. PF7508-250H363), using a binary buffer system consisting of solution A: 0.1% formic acid and B: 80% acetonitrile, 0.1% formic acid. Runs of 120 min, after loading, were used for proteome samples, with a constant flow rate of 300nl/min. After sample loading, run start at 5% buffer B for 5min, followed by a series of linear gradients, from 5% to 30% B in 90min, then a 10 min step to reach 50% and a 5 min step to reach 95%. This last step was maintained for 10 min.

Q Exactive HF settings: MS spectra were acquired using 3E6 as an AGC target, a maximal injection time of 20ms and a 120,000 resolution at 200m/z.

The mass spectrometer operated in a data dependent Top20 mode with sub sequent acquisition of higher-energy collisional dissociation (HCD) fragmentation MS/MS spectra of the top 20 most intense peaks. Resolution, for MS/MS spectra, was set to 15,000 at 200m/z, AGC target to 1E5, max injection time to 20ms and the isolation window to 1.6Th. The intensity threshold was set at 2.0 E4 and Dynamic exclusion at 30 seconds.

All acquired RAW files were processed using MaxQuant (1.6.2.10) and the implemented Andromeda search engine. For protein assignment, spectra were correlated with the UniProt mouse database (v. 2019) including a list of common contaminants. Searches were performed with tryptic specifications and default settings for mass tolerances for MS and MS/MS spectra. Carbamidomethyl at cysteine residues was set as a fixed modification, while oxidations at methionine, acetylation at the N-terminus were defined as variable modifications. The minimal peptide length was set to seven amino acids, and the false discovery rate for proteins and peptide-spectrum matches to 1%. For label free quantification (LFQ), minimum ratio count was set as 1. The match-between-run feature with a time window of 1 min was used. For further analysis, the Perseus software (1.6.2.3) was used and first filtered for contaminants and reverse entries as well as proteins that were only identified by a modified peptide. The LFQ Ratios were logarithmized, grouped and filtered for min. valid number (min. 4 in at least one group). Missing values have been replaced by random numbers that are drawn from a normal distribution. Two sample t-test was performed using FDR=0.05. Probability values (p) < 0.05 were considered statistically significant. The mass spectrometry proteomics data have been

deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020385.

Electron microscopy and DAB staining

Cells were fixed in a 24 wells plate with 4% Paraformaldehyde in PBS (pH 7,4) for 30 min. at RT. After fixation cells were washed 5 times with PBS (5 min. each) blocked and permeabilized with 5% normal goat serum and 0,1% saponin in PBS for 30 min, and then incubated with primary antibody anti-Stat3 O.N at 4°C. in PBS 5% normal goat serum and 0,05% saponin. After 5 washing with PBS, (5 min each) cells were incubated with HRP-conjugated Fab fragments of the secondary antibody for 2h. RT. After 5 washing cells were incubated in the DAB solution (0.01gr DAB in 20ml TRIS-HCl buffer plus 30% H2O2 solution just before use). Subsequently the samples were postfixed with 1% osmium tetroxide plus potassium ferrocyanide 1% in 0.1M sodium cacodylate buffer for 1 hour at 4°. After three water washes, samples were dehydrated in a graded ethanol series and embedded in an epoxy resin (Sigma-Aldrich). Ultrathin sections (60-70 nm) were obtained with an Ultrotome V (LKB) ultramicrotome, counterstained with uranyl acetate and lead citrate and viewed with a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera.

Statistics and reproducibility

For each dataset, sample size n refers to experimental or biological replicates, as stated in the figure legends. All *P* values were calculated using the unpaired two-tailed T-test and indicated as their numerical values in each plot; *P* values were not calculated for datasets with $n < 3$.

Either Excel or R software were used for statistical analysis. Error bars indicate the standard error of the mean (s.e.m.) or the standard deviation (SD), as stated in the figure legends.

Fig. S1.

Fig. S1. LIF/Stat3 induced hypomethylation in mES cells:

a, Histograms showing the distribution of DNA methylation levels at CpG islands measured by RRBS in S3+/+ cells cultured in Serum LIF, 2i or 2iLIF and S3-/- cells in 2iLIF.

b, Histograms showing expression (RPKM) of 7 genes involved in DNA methylation and methylcytosine oxidation in RGd2 cells expanded in 2i media with or without LIF. Socs3 was used as a control of LIF/Stat3 activation. Bars represent means +/- SD of n = 2 independent biological replicates.

c, Volcano plot of proteomics data, depicting differences in protein abundances between S3-/- and S3+/+ cells cultivated in 2iLIF. Each dot represents one protein. The x axis shows the fold change (FC) in protein abundance (measured as LFQ intensity, in log scale) and the y axis represents the statistical significance (adjusted *P* value, in log-scale). Yellow and blue dots indicate respectively proteins that are less or more (FC > 1 or FC < -1 respectively, *P* value < 0.05) in S3-/- cells with respect to S3+/+ cells. All proteomics data are in Supplementary table 7.

d, Western blot of E14 mES cells and of Dnmt3a KO, Dnmt3b KO, and Dnmt3a/b double KO mES cells (two clones per genotype) cultivated in Serum LIF. B-ACTIN was used as a loading control.

e, Histograms showing the distribution of DNA methylation levels at CpG islands measured by RRBS in E14 mES cells cultured in 2i or 2iLIF and two clones of Dnmt3a/b double KO mES cells in 2i.

f, Gene expression analysis by RT–qPCR of S3+/+ cells cultured in 2iLIF transiently expressing an Empty Vector , Dnmt3a (two isoforms, Dnmt3a1 and Dnmt3a2 – as previously identified in 17,56), Dnmt3b, or the three genes simultaneously (Dnmt3a1/a2/b OE). Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n = 3 independent transfection experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to S3+/+ 2iLIF Empty Vector.

g, Immunofluorescence for 5mC on S3+/+ cultured in 2i and 2iLIF, and on Dnmt3a1 OE, Dnmt3a2 OE, Dnmt3b OE, and Dnmt3a1/a2/b triple OE mES cells cultured in 2iLIF. Top: Violin plots show the distribution of fluorescence intensity of an average of 89 nuclei per sample, normalized to the mean intensity of S3+/+ 2iLIF Empty Vector. Boxplots show 1st, 2nd and 3rd quartile; whiskers indicate minimum and maximum values. n = 4 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the 6 conditions in analysis. Scale bar: 20µm.

h, Gene expression analysis by RT–qPCR of S3+/+ cells cultured in 2iLIF stably expressing a shRNAs to knock-down Tet1 and Tet2 simultaneously or a scrambled control shRNA. Beta-actin serves as an internal control. Bars indicate mean and s.e.m. of n = 4 independent knock-down experiments, shown as dots. *P* values calculated using two-tailed unpaired T-test relative to S3+/+ 2iLIF sh-Scramble.

i,Immunofluorescence for 5mC on S3+/+ cultured in 2iLIF transiently expressing a scrambled control shRNA and a shRNAs against Tet1/Tet2. Left: Violin plots show the distribution of fluorescence intensity of an average of 78 nuclei per sample, normalized to the mean intensity of scrambled control shRNA**.** Boxplots show 1st, 2nd and 3rd quartile; Whiskers indicate minimum and maximum values. n = 4 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Right: representative images of S3+/+, S3-/-, sh-Scramble and sh-Tet1/2 cell lines cultivated in 2iLIF. Scale bar: 20µm.

Fig. S2.

Fig. S2

a, Representative confocal images of S3-/- cells in 2i+TAM (control) and one representative clone of S3ER cells cultured either in 2i or 2i+TAM (1mM), stained with anti-Stat3 antibody. S3ER cells are S3-/- cells that were transfected with Stat3 conjugated with Estrogen Receptor (ER). Upon induction with Tamoxifen, this construct is re-targeted from cytoplasm to nucleus. DAPI serves as a nuclear counterstain. Scale bar: 20μm.

b, Gene expression analysis by RT–qPCR of S3+/+ 2iLIF, S3-/- 2iLIF, and three independent S3ER clones (S3ER.A, S3ER.B, S3ER.C) grown in 2i with or without Tamoxifen. Beta-actin serves as an internal control. Bars indicate mean and s.e.m. of n = 3 independent experiments for each S3ER clone, shown as dots, squares or triangles. *P* values calculated using two-tailed unpaired T-test.

c, Representative confocal images S3+/+ and S3-/- cells in 2iLIF and one representative clone of S3ER cells cultured either in 2i or 2i+TAM, stained with anti-5mC and anti-Dnmt3b antibodies. DAPI serves as a nuclear counterstain. Scale bar: 20μm.

d, Histogram showing quantification of fluorescence intensity in S3+/+ 2iLIF, S3-/- 2iLIF, and three independent S3ER clones (S3ER.A, S3ER.B, S3ER.C) grown in 2i with or without Tamoxifen, stained with anti-Dnmt3b (left) and anti-5mC (right) antibodies. Bars indicate mean and s.e.m. n = 3 independent experiments for S3+/+ 2iLIF and S3-/- 2iLIF cells, and n = 3 independent experiments for each S3ER clone, shown as dots, squares or triangles. *P* values calculated using two-tailed unpaired T-test relative to S3-/- 2iLIF.

Fig. S3.

Fig. S3. Impact of Stat3 on repetitive elements and imprinted regions:

a, MeDIP-qPCR analysis of repetitive elements in S3+/+ and S3-/- cells. Real-time qPCR was carried out on enriched methylated DNA fractions ("IP") immunoprecipitated from genomic DNA with an antibody specific for 5mC (MeDIP). Negative controls were included, where a non-specific antibody (IgG) was used for immunoprecipitation ("mock"). Enrichment was calculated as % of Input and mean normalised for each experiment. Bars represent the mean and SD of n = 2 independent MeDIP experiments, shown as dots.

b, Scatter plot comparing effects on transcription (RNA-seq data) of the absence of LIF (S3+/+ 2i) and the absence of Stat3 (S3-/- 2iLIF). For each gene, the x axis reports the fold change in gene expression between S3-/- and S3+/+ cells in 2iLIF (logarithmic scale), and the y axis the fold change in gene expression between S3+/+ 2i and S3+/+ 2iLIF. Some genes of interest, Pearson's correlation coefficient (R) and corresponding *P* value are indicated in the panel; high correlation coefficient suggests that absence of LIF or of Stat3 have overlapping effects on gene expression.

c, Volcano plot showing the significant differentially methylated CpG sites between E14 2i and E14 2iLIF cells (**Q-value < 0.01, abs(average methylation difference) > 10%**). The x and y axis represent the difference in methylation levels (reported as percentage) and the statistical significance (in log-scale) respectively. Red dots depict hyper-methylated sites in E14 2i cells, blue dots the hypo-methylated ones. The analysis confirmed the overall gain in DNA methylation levels for cells cultured in 2i as compared to 2iLIF in an independent cell line (E14), with 481,279 hyper-methylated CpG sites and only 355 hypo-methylated sites out of a total of **xxx**sites mapped.

d, Volcano plot showing the significant differentially methylated CpG sites between Dnmt3a dKO clone A and E14 cells cultivated in 2i, as described in panel S3c.

e, Venn diagram showing number of CpG sites whose methylation status is dependent on LIF (light blue circle) or on Dnmt3a/b (red circle) for an independent mutant Dnmt3a/b dKO clone (Dnmt3a/b dKO.B), as described in Fig. 2h.

f, Scatter plot comparing the effects of adding LIF and deleting Dnmt3a/b on the levels of CpG methylation in wild-type E14 cells for an independent mutant dKO clone (Dnmt3a/b dKO.B), as described in Fig. 2i.

g, Volcano plot showing the significant differentially methylated CpG sites between Dnmt3a dKO clone B and E14 cells cultivated in 2i, as described in panel S3c.

h, MeDIP-qPCR analysis of two different promoters region: Gapdh as a control (methylation is unchanged) and H19 that is a positive control (methylation is increased in S3-/- cells). Enrichment was calculated as % of Input and mean normalised for each experiment. Bars represent mean and s.e.m. of n = 4 independent MeDIP experiments, shown as dots.

i, Differentially Methylated Regions and associated imprinted genes. Top: table reports Differentially Methylated Regions (DMRs) analysed by RRBS with associated coordinates (reference genome: mm10); information about these DMRs in mouse genome was collected from three different databases (WAMIDEX https://atlas.genetics.kcl.ac.uk/; MouseBook - Imprinting Locihttps://www.mousebook.org/; Geneimprint http://geneimprint.com/site/genes-by-species.
Imprinted genes whose expression is controlled by the same DMR are grouped accordingly and indicated in the second column of the table. For each imprinted gene, fourth column reports expected effect on gene expression - either upregulation or downregulation - caused by methylation deposition at the associated DMR (data from literature). Last column of the table shows observed expression levels (RNAseq data) of each imprinted gene in S3-/- cells, were hypermethylation was detected at the corresponding DMR (see Fig. 2l).

j, Histogram showing correlation between differential DNA methylation and gene expression at promoters, enhancers and imprinted DMRs (see Fig. 2b, c, j, l). Panel reports the percentage of differentially expressed (DE) genes associated with known DMRs (Fig. 2j), in the three specific genomic regions. *P* value derived by hypergeometric test to evaluate the over-representation of DE genes associated with DMR compared to total DE genes (see Fig. 2m).

Fig. S4.

Fig. S4. Stat3 controls DNA methylation via metabolic regulation:

a, Immunofluorescence on S3+/+ cells cultured in 2i or 2iLIF and S3-/- cells in 2iLIF stained with anti-5mC antibody and treated with EdU (5-ethynyl-2'-deoxyuridine is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis) for 4 h. Left: Violin plots show the distribution of fluorescence intensity of an average of 67 nuclei per sample, normalized to S3+/+ 2iLIF EdU +; boxplots show 1st, 2nd and 3rd quartile; one representative experiment is shown for each condition. Whiskers indicate minimum and maximum values. Note that EdU positive cells (in which genome replication occurred during the 4 hour-treatment) show a decrease in 5mC compared to EdU negative cells, which never reaches the levels of S3+/+ in 2iLIF. Right: representative images of the conditions in analysis.

b, Immunofluorescence staining of 5mC in S3+/+ cells treated with respiratory chain Complex I inhibitor Rotenone or Complex III inhibitor Antimycin A. Top: Violin plots show the distribution of fluorescence intensity of an average of 74 nuclei per sample, normalized to the mean intensity of S3+/+ 2iLIF. Boxplots show 1st, 2nd and 3rd quartile. Whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the 3 conditions in analysis. Scale bar: 20μm.

c, Immunofluorescence for 5mC of E14 cultured in 2iLIF and 2i, and of Dnmt3a/b double KO mES cells cultured in 2iLIF; each cell line was treated with either Vehicle, Rotenone (100nM) or Antimycin (200nM). Top: Violin plots show the distribution of fluorescence intensity of an average of 183 nuclei per sample, normalized to the mean intensity of E14 2iLIF + Vehicle. Boxplots show 1st, 2nd and 3rd quartile; whiskers indicate minimum and maximum values. n = 3 independent experiments are shown as individual violins. Two-tailed unpaired T-test was performed on median intensity values of each sample. Bottom: representative images of the conditions in analysis. Scale bar: 20μm.

d, Representative confocal images of S3+/+, S3-/- cells and two MitoS3.A/B clones stained with anti-Stat3 and anti-Atad3 antibodies. Merged images show colocalization between Stat3 and the nucleoids marker Atad3. DAPI serves as a nuclear counterstain. Scale bar: 20μm.

e, Representative Electron Microscopy images of STAT3 protein stained by Diaminobenzidine (DAB) photooxidation method, in Stat3-/- and MitoS3.A cells (see Methods). Representative images are shown at the same magnification. M, mitochondria; N, nucleus.

f, Expression analysis of Socs3, a nuclear target of Stat3, by RT–qPCR in S3+/+, S3-/- cells and two MitoS3.A/B clones cultured in 2iLIF. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean +/- SD of n = 2 independent experiments, shown as dots. Note that mitochondrial Stat3 (MitoS3.A/B) does not affect the expression of Socs3. Similar results were obtained by RNAseq (see Supplementary table 4).

g, Western blot of S3+/+, S3-/- cells and in MitoS3.A and MitoS3.B clones cultured in 2iLIF. MitoS3 clones were generated by stable transfection of S3-/- with a piggyBac vector containing a mouse Stat3 cDNA tagged with a MLS (Mitochondrial Localization Signal) and NES (Nuclear Export Signal) to ensure mitochondrial localisation and absence of nuclear transcriptional activity. Note the shift in molecular weight due to the presence of MLS and NES tag. Stat3 protein level in both MitoS3 clones is lower than in S3+/+ cells. LAMIN B was used as a loading control.

h, Oxygen consumption rate (OCR) measured by Seahorse extracellular flux assay of S3+/+, S3-/- and two MitoS3.A/B clones cultured in 2iLIF. MitoS3.A/B clones show significant increase in the respiratory capacity, both at the basal level and post FCCP (mitochondrial uncoupler) treatment. OCR data are normalized to the amount of protein for each well. Mean and SD of n=5 technical replicates is shown.

Fig. S5.

Fig. S5.

a, Metabolic tracing analysis of Alpha-ketoglutarate (αKG), Oxaloacetate (OAA) and Fumarate using [U-$^{13}C_6$]-Glucose, [U-$^{13}C_5$]-Glutamine or [U-13C16]-Palmitate. Histogram represents mole percent enrichment (MPE), i.e. the percentage of marked carbon within the different metabolites, after provision of the marked carbon source to cultured cells for 2h, 4h and 8h. Note that MPE values were constant over the 3 time points used, indicating that 13C-labelling has reached the isotopic equilibrium, as previously reported [30]. Each bar represents mean +/- s.e.m of n = 6 biological replicates.

b, Diagram representing mass isotopomer distribution (MID) of αKG in TCA; MID was analysed following 8h of metabolic tracing with [U-$^{13}C_5$]-Glutamine. Color scale outlines the comparison between MID profile in S3-/- cells as compared to S3+/+ for n = 6 biological replicates, where blue color indicates isotopomers (or biochemical pathways) under-represented is S3-/- cells and red color isotopomers or pathways over-represented in S3-/- cells with respect to S3+/+ cells.

c, Metabolic tracing analysis of different isotopomers of TCA cycle intermediates (Succinate, Oxaloacetate, Citrate and αKG) using [U-$^{13}C_5$]-Glutamine. Histogram represents mass isotopomer distribution (MID) at 3 different time points (2h, 4h, 8h). Each bar represents mean +/- s.e.m of n = 6 biological replicates. * indicates a p-value<0.05, two-tailed unpaired T-test. Each isotopomer is corrected for natural isotope abundances.

d, Histogram reporting expression of IDH1, a cytosolic enzyme involved in reductive Glutamine metabolism, which displays decreased expression in S3-/- cells relative to S3+/+. Relative mRNA expression (left) measured by RT q-PCR and TPM (right) measured by RNAseq are shown for S3+/+ and S3-/- cells. Bars represent means +/- SD of n = 2 independent biological replicates.

e, Gene expression analysis of two Hif1a targets by RT–qPCR of S3+/+ cells cultured in 2iLIF in normoxia (high 21% O2), in hypoxia (low 1% O2), or in normoxia with the addition of αKG or DM-αKG. Beta-actin serves as an internal control. Expression values were mean-normalised. Bars indicate mean and s.e.m. of n = 7 biological replicates for high O2 and low

O2 conditions, and n = 4 biological replicates for treatments with αKG and DM-αKG, shown as dots.

Fig. S6

Fig. S6

a, Mass Spectrometry showing percentages of h5mC in the DNA of S3+/+, S3-/- cells and two MitoS3.A/B clones, cultured in 2iLIF. h5mC content is expressed as the percentage of h5mC in the total pool of cytosines. Mass Spectrometry shows that mitochondrial Stat3 is associated with increased levels of h5mC. Bars indicate mean +/- s.e.m. of n = 4 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test.

b, Western blot of S3+/+, S3-/- cells and two MitoS3.A/B clones cultured in the presence of 2iLIF (left). *De novo*DNA methyltransferases DNMT3A (90 and 130 kDa) and DNMT3B (130 kDa) were less abundant in cells expressing mitochondrial Stat3, compared to S3-/- cells. As a control, cells were exposed to differentiation in basal medium for 24h (right). As previously reported (17,36), DNMT3A/B were upregulated. LAMIN B was used as a loading control.

c, Histogram showing quantification by Mass Spectrometry of αKG abundance in S3+/+ cells and in S3-/- cells treated with vehicle or DM- αKG for 24h (dark bars) or for 3 passages (light bars). Bars indicate mean +/- s.e.m. of n = 6 biological replicates, shown as dots. *P* values calculated using two-tailed unpaired T-test

d, Heatmap reporting expression of 3 regulators of Dnmt3a and Dnmt3b enzymes. RNAseq data derived from S3+/+, S3-/-, MitoS3.A and MitoS3.B cells; n = 2 biological replicates are reported for each condition. Expression levels were scaled and represented as z-score.

e, Heatmap reporting expression of Dnmt3a, Dnmt3b and Otx2 in S3+/+ cultured in 2i with or without LIF. n = 2 biological replicates are reported for each condition. Expression levels were scaled and represented as z-score.

Fig. S7.

Fig. S7. Mitochondrial Stat3 regulates expression of imprinted genes and germ layer markers: Gene expression analysis by RT–qPCR of S3+/+ (blue), S3-/- (red) and two MitoS3.A/B (orange) clones cultured with 2iLIF or without 2iLIF for 24h or 48h. Data show expression of Imprinted genes **a**, Mesoderm **b**, Ectoderm **c**, and PGCs **d,** markers that are more readily induced in S3-/- and MitoS3.A/B clones rescues this effect. Data are normalized to the mean of 3 independent experiments. Beta-actin served as an internal control. Bars indicate mean +/- s.e.m. of n = 3 independent experiments, shown as dots. Two-tailed unpaired T-test relative to S3-/- for each time point.

Fig. S8.

Fig. S8. Mitochondrial Stat3 regulates expression of genes associated to differentially methylated elements: a, Left: Boxplot reporting expression levels of genes down-regulated in S3-/- cells with respect to S3+/+ cells (Fig. 5a, blue dots) and with differential methylation in promoter regions (Fig. 2b). Each boxplot shows 1st, 2nd and 3rd quartile. Whiskers shows minimum and maximum values. Y axis represents mean-normalized TPM values for S3+/+, S3-/- and Mito-Stat3 clones (MitoS3.A and MitoS3.B) in two different conditions: following stable culturing of cells in 2iLIF (light blue) and after 48h of 2iLIF withdrawal from culture medium (dark blue). Right: Boxplot reporting expression levels of genes up-regulated in S3-/- cells with respect to S3+/+ cells (Fig. 5a, yellow dots) and with differential methylation in promoter regions (Fig. 2b). Each boxplots shows 1st, 2nd and 3rd quartile. Whiskers shows minimum and maximum values. Y axis represents mean-normalized TPM for S3+/+, S3-/- and Mito-Stat3 clones (MitoS3.A and MitoS3.B) in two different conditions: following stable culturing of cells in 2iLIF (light yellow) and after 48h of 2iLIF withdrawal from culture medium (dark yellow). **b,** Boxplot reporting expression levels of genes down-regulated (left) or up-regulated (right) in S3-/- cells with respect to S3+/+ cells (Fig. 5a, blue and yellow dots respectively) and with differential methylation in enhancer regions (Fig. 2c), as described above. **c,** Boxplot reporting expression levels of genes down-regulated (left) or up-regulated (right) in S3-/- cells with respect to S3+/+ cells (Fig. 5a, blue and yellow dots respectively) and with differential methylation at the associated DMR (Fig. 2g), as described above. **d,** Merge of genes contained in boxplots shown in panels S8a,b,c.

Fig. S9.

Fig. S9. Stat3 regulates PrE and post implantation epiblast transcripts in the early mouse blastocysts: a, Diffusion pseudotimes of E3.5 cells and the PrE gene signature were computed with R package "destiny" (http://bioinformatics.oxfordjournals.org/content/32/8/1241) using all the expressed genes as input list. Each boxplot shows 1st, 2nd and 3rd quartile. Whiskers shows minimum and maximum values. Mann-Whitney test was used to compute the p-value. **b,** PCA plot computed with all the expressed genes. Colors represent different lineages/genotypes (left panel) or ratio between Gata6 and Nanog expression (right panel).
c, Fraction of identity between E3.75 EPI(left panel)/PrE(right panel) and E3.5 ICM, E4.5 EPI, E4.5 PrE, E5.5 EPI and E6.5 EPI stages computed with all the expressed genes. Each boxplot shows 1st, 2nd and 3rd quartile. Whiskers shows minimum and maximum values. Mann-Whitney test was used to compute the p-value. (See also S9d for additional analysis).
d, Boxplot of log2FC between E3.75 WT and MUT computed using the top 100 ranked differentially expressed genes between E4.5 EPI (red) and PrE (purple). Each boxplots shows 1st, 2nd and 3rd quartile. Whiskers shows minimum and maximum values. Mann-Whitney test was used to compute the p-value.

Supplementary Table 1. Specifications of antibodies used for immunostaining.

Antibody	Species	Source	Dilution	Antibody validation
5mC	Mouse monoclonal	EUROGENTEC BI-MECY	IF: 1:250	Previously validated in M. Habib 1999 Experimental Cell Research[85] Results obtained were confirmed by Mass Spectrometry for 5mC
Tet2	Rabbit polyclonal	ABCAM Ab94580	WB: 1:500	Previously validated in Neri 2015 Cell Reports [86]
Dnmt3a	Mouse monoclonal	Novus Biological NB120-13888	WB: 1:500	Previously validated in Neri 2015 Cell Reports [86]
Dnmt3b	Mouse monoclonal	Novus Biological NB100-56514	WB: 1:1000	Previously validated in Neri 2015 Cell Reports [86]
LaminB	Goat polyclonal	Santa Cruz Biotechnologies cat. 6216	WB: 1:1,000	Previously validated in Su 2013 Molecular Cell Biology [87]
Stat3	Mouse monoclonal	Cell Signalling cat. 9139	WB: 1:1,000 IF: 1:100	Previously validated in Carbogning 2016 EMBOJ[19]. Signal is absent in Stat3-/- cells.
Atad3	Rabbit monoclonal	AB-Biotechnologies cat. 224485	IF 1:100	Previously validated in He 2007 Journal of cell biology [88]

Supplementary Table 2. Primers used for Real-time RT q-PCR.

Gene RT-qPCR	Forward primer sequence	Reverse primer sequence
Socs3	ATTTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT
Uhrf1	GCTCCAGTGCCGTTAAGACC	CACGAGCACGGACATTCTTG
Dnmt3l	ATGGACAATCTGCTGCTGACTG	CGCATAGCATTCTGGTAGTCTCTG
Tet1	GAGCCTGTTCCTCGATGTGG	CAAACCCACCTGAGGCTGTT
Tet2	TGTTGTTGTCAGGGTGAGAATC	TCTTGCTTCTGGCAAACTTACA
Dnmt1	CCATGGCTGACACTAAGCTG	ACCAAACCAAACCAAACCAA
Dnmt3b 3'UTR	CTCGCAAGGTGTGGGCTTTTGTAAC	CTGGGCATCTGTCATCTTTGCACC
Dnmt3a 3'UTR	GACTCGCGTGCAATAACCTTAG	GGTCACTTTCCCTCACTCTGG
Idh1	ATGGGCGTTTCAAAGACATC	CCTCGGACTTCATAGCTTGG
Peg10	ACGATGATGACCTGGAGCTT	ATGAAAGGACCCAGCATGTC
Phlda2	TCAGCGCTCTGAGTCTGAAA	CTCCTGGGCTCCTGTCTGAT
Lin28a	GTCTTTGTGCACCAGAGCAA	CGCTCACTCCCAATACAGAA
Lin28b	ACGGCAGGATTTACTGATGG	GCACTTCTTTGGCTGAGGAG

Oct6/Pou3f1	ACCACCACCACCACACACT	AAATCCAAAGCAAAACCGAAT
Cdkn1c	GGAGCAGGACGAGAATCAAG	GTTCTCCTGCGAGTTCTCT
Sfmbt2	CATGTGGAGATCAGCATTCG	TGTCCACAGGTGGTGATGAT
Igf2	GACGACTTCCCCAGATACCC	CTTTGAGCTCTTTGGCAAGC
Brachyury	CTGGGAGCTCAGTTCTTTCGA	GAGGACGTGGCAGCTGAGA
Hand1	AGAGGAGACGCACAGAGAGC	AGCACGTCCATCAAGTAGGC
Snai	TGAGAAGCCATTCTCCTGCT	CTTCACATCCGAGTGGGTTT
Tubb3	CATGGACAGTGTTCGGTCTG	CGCACGACATCTAGGACTGA
Krt18	CGAGGCACTCAAGGAAGAAC	GCTGAGGTCCTGAGATTTGG
Nes	CTGCAGGCCACTGAAAAGTT	TTCCAGGATCTGAGCGATCT
Tfcp2l1	GGGGACTACTCGGAGCATCT	TTCCGATCAGCTCCCTTG
Tbx3	TTGCAAAGGGTTTTCGAGAC	TGCAGTGTGAGCTGCTTTCT
Dppa3	TTTGTTGTCGGTGCTGAAAG	TCATTTCCTTCGAGCCTTTT
Beta-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Dnmt3a1	GAAGGCCGTGGAGCCTCT	CCATTTTCATGGATTCGATGTT
Dnmt3a2	AGAAGTGTCTGCTGCACACA	TGCTCCAGACACTCTTGCAG
Dnmt3b INT	TGACGTCCGGAAAATCACCA	TAAACCTTTGCGGGCAGGAT
Otx2	GGAAGAGGTGGCACTGAAAA	CGGCACTTAGCTCTTCGATT

Sox1	CACAACTCGGAGATCAGCAA	CTCGGACATGACCTTCCACT
Klf4	CGACTAACCGTTGGCGTGA	CGGGTTGTTACTGCTGCAAG
Tbx3	TTGCAAAGGGTTTTCGAGAC	TGCAGTGTGAGCTGCTTTCT
Nanog	TTCTTGCTTACAAGGGTCTGC	AGAGGAAGGGCGAGGAGA
Prdm14	GAAGGCACACAGGGACAACT	TCCAGTTCCCAGAACCTTTG
Tcl1	GATCTGGGAGAAGCACGTGTA	TGACTGGGGGACATAGCTTC
Tcea3	GACAAGCTGGCCTCAGAAAT	CGGTTCCGGTACTTCATGTC
Vegfa	CTGTAACGATGAAGCCCTGGAG	TGGTGAGGTTTGATCCGCAT
Phd3	CTTCCTCCTGTCCCTCATCG	ATACAGCGGCCATCACCATT

Supplementary Table 3. Primers used for MeDIP-PCR.

Gene MeDIP-qPCR	Forward primer sequence	Reverse primer sequence
Nnat	GAGTATGTACCCGGGCTTTG	ATAGGATGGGTTGGGTAGGG
Peg10	CCCCCTCCTAGGATCTCTCT	GGATTCTTCGACACACACCA
Kif27	CCAGCTGAGGGGATAACTCA	TCTGGGTCCTTTCAATACCAA
Iap	CTCCATGTGCTCTGCCTTCC	CCCCGTCCCTTTTTTAGGAGA

Iapey3	AGAGAGGAGGACAACTGCTC	AACCTTACACAGGCAAAAGC
Line L1	CTGGCGAGGATGTGGAGAA	CCTGCAATCCCACCAACAAT
MajSat	GACGACTTGAAAAATGACGAAATC	CATATTCCAGGTCCTTCAGTGTGC
MinSat	CATGGAAAATGATAAAAACC	CATCTAATATGTTCTACAGTGTGG
Sine B1	GTGGCGCACGCCTTTAATC	GACAGGGTTTCTCTGTGTAG
Sine B2	GAGATGGCTCAGTGGTTAAG	CTGTCTTCAGACACTCCAG
Gapdh	TCCCTAGACCCGTACAGTGC	CTCTGCTCCTCCCTGTTCC
Zdbf2	CCAAACCCATCTCCTCTTCA	TGGCCTGGTCTAGTCGTCTC
H19	GCATGGTCCTCAAATTCTGCA	GCATCTGAACGCCCCAATTA

Supplementary Table 4. (separate file)

Table reports bulk RNA sequencing data, including differential expression analysis of following comparisons: S3-/- cells vs S3+/+ cells (Figure 5a), MitoS3.A cells vs S3-/- cells and MitoS3.B cells vs S3-/- cells. Table also reports absolute expression data (TPM) of : S3+/+ cells in 2i; S3+/+, S3-/- , MitoS3.A and MitoS3.B cells in 2iLIF; S3+/+, S3-/- , MitoS3.A and MitoS3.B cells without 2iLIF for 48h.

Supplementary Table 5. (separate file)

Table reports the differential expression analysis (RNA-seq) and RRBS analysis of S3-/- vs S3+/+ cells, for the following genomic features: promoters; enhancers; imprinted DMRs.

Supplementary Table 6. (separate file)

This table reports the differential expression analysis (single cell RNA-seq) for the following samples: E3.5 S3-/- vs E3.5 S3+/+ cells; E3.75 S3-/- vs E3.75 S3+/+ cells. Table also reports absolute expression levels (FPKM) of selected genes.

Supplementary Table 7. (separate file)

This table reports Mass spectrometry proteomics data of following samples: S3+/+ cells in 2i; S3+/+, S3-/- , MitoS3.A and MitoS3.B cells in 2iLIF

ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD020385

References

1. Ito, S. *et al.* Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129–1133 (2010).
2. Ito, S. *et al.* Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**, 1300–1303 (2011).
3. Messerschmidt, D. M., Knowles, B. B. & Solter, D. DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes Dev.* **28**, 812–828 (2014).
4. Monk, M., Boubelik, M. & Lehnert, S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Dev. Camb. Engl.* **99**, 371–382 (1987).
5. Smith, Z. D. *et al.* A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* **484**, 339–344 (2012).
6. Ishida, M. & Moore, G. E. The role of imprinted genes in humans. *Mol. Aspects Med.* **34**, 826–840 (2013).
7. Boroviak, T. *et al.* Lineage-Specific Profiling Delineates the Emergence and Progression of Naive Pluripotency in Mammalian Embryogenesis. *Dev. Cell* **35**, 366–382 (2015).
8. Do, D. V. *et al.* A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. *Genes Dev.* **27**, 1378–1390 (2013).
9. Martello, G., Bertone, P. & Smith, A. Identification of the missing pluripotency mediator downstream of leukaemia inhibitory factor. *EMBO J.* **32**, 2561–2574 (2013).
10. Mohammed, H. *et al.* Single-Cell Landscape of Transcriptional Heterogeneity and Cell Fate Decisions during Mouse Early Gastrulation. *Cell Rep.* **20**, 1215–1228 (2017).
11. Ye, S., Li, P., Tong, C. & Ying, Q. L. Embryonic stem cell self-renewal pathways converge on the transcription factor Tfcp2l1. *EMBO J.* **32**, 2548–2560 (2013).

12. Boroviak, T. & Nichols, J. Primate embryogenesis predicts the hallmarks of human naïve pluripotency. *Development* **144**, 175–186 (2017).
13. Ying, Q.-L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519–523 (2008).
14. Ficz, G. *et al.* FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency. *Cell Stem Cell* **13**, 351–359 (2013).
15. Habibi, E. *et al.* Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* **13**, 360–369 (2013).
16. Hackett, J. A. *et al.* Synergistic mechanisms of DNA demethylation during transition to ground-state pluripotency. *Stem Cell Rep.* (2013) doi:10.1016/j.stemcr.2013.11.010.
17. Leitch, H. G. *et al.* Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol.* **20**, 311–316 (2013).
18. Smith, A. G. *et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690 (1988).
19. Carbognin, E., Betto, R. M., Soriano, M. E., Smith, A. G. & Martello, G. Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency. *EMBO J.* **35**, 618–634 (2016).
20. Gough, D. J. *et al.* Mitochondrial STAT3 supports RasDependent oncogenic transformation. *Science* **324**, 1713–1716 (2009).
21. Wegrzyn, J. Function of Mitochondrial Stat3 in Cellular Respiration Joanna Wegrzyn,. **793**, 793–798 (2009).
22. Lu, C. & Thompson, C. B. Metabolic regulation of epigenetics. *Cell Metab.* **16**, 9–17 (2012).
23. Kalkan, T. *et al.* Tracking the embryonic stem cell transition from ground state pluripotency. *Dev. Camb. Engl.* **144**, 1221–1234 (2017).
24. Dawlaty, M. M. *et al.* Loss of Tet Enzymes Compromises Proper Differentiation of Embryonic Stem Cells. *Dev. Cell* **29**, 102–111 (2014).
25. Sánchez-Castillo, M. *et al.* CODEX: a next-generation sequencing experiment database

for the haematopoietic and embryonic stem cell communities. *Nucleic Acids Res.* **43**, D1117–23 (2015).

26. Elhamamsy, A. R. Role of DNA methylation in imprinting disorders: an updated review. *J. Assist. Reprod. Genet.* **34**, 549–562 (2017).
27. Ferguson-Smith, A. C. Genomic imprinting: the emergence of an epigenetic paradigm. *Nat. Rev. Genet.* **12**, 565–575 (2011).
28. Hackett, J. A., Kobayashi, T., Dietmann, S. & Surani, M. A. Activation of Lineage Regulators and Transposable Elements across a Pluripotent Spectrum. *Stem Cell Rep.* **8**, 1645–1658 (2017).
29. Peron, M. *et al.* Mitochondrial STAT3 regulates proliferation of tissue stem cells. *bioRxiv* 2020.07.17.208264 (2020) doi:10.1101/2020.07.17.208264.
30. Carey, B. W., Finley, L. W. S., Cross, J. R., Allis, C. D. & Thompson, C. B. Intracellular α-ketoglutarate maintains the pluripotency of embryonic stem cells. *Nature* **518**, 413–416 (2015).
31. Hou, P. *et al.* Intermediary Metabolite Precursor Dimethyl-2-Ketoglutarate Stabilizes Hypoxia-Inducible Factor-1α by Inhibiting Prolyl-4-Hydroxylase PHD2. *PLOS ONE* **9**, e113865 (2014).
32. Tischler, J. *et al.* Metabolic regulation of pluripotency and germ cell fate through α-ketoglutarate. *EMBO J.* **38**, e99518 (2019).
33. Laukka, T. *et al.* Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes via TET Enzymes. *J. Biol. Chem.* **291**, 4256–4265 (2016).
34. Xiao, M. *et al.* Corrigendum: Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. *Genes Dev.* **29**, 887–887 (2015).
35. Teslaa, T. & Teitell, M. A. Pluripotent stem cell energy metabolism: an update. *EMBO J.* **34**, 138–153 (2015).
36. Nishiyama, A. *et al.* Systematic repression of transcription factors reveals limited patterns of gene expression changes in ES cells. *Sci. Rep.* **3**, 5–10 (2013).

37. Correa-Cerro, L. S. *et al.* Generation of mouse ES cell lines engineered for the forced induction of transcription factors. *Sci. Rep.* **1**, 1–6 (2011).
38. Nishiyama, A. *et al.* Uncovering Early Response of Gene Regulatory Networks in ESCs by Systematic Induction of Transcription Factors. *Cell Stem Cell* **5**, 420–433 (2009).
39. Buecker, C. *et al.* Reorganization of enhancer patterns in transition from naive to primed pluripotency. *Cell Stem Cell* **14**, 838–853 (2014).
40. Yang, S.-H. *et al.* Otx2 and Oct4 Drive Early Enhancer Activation during Embryonic Stem Cell Transition from Naive Pluripotency. *Cell Rep.* **7**, 1968–1981 (2014).
41. Yamaji, M. *et al.* PRDM14 Ensures Naive Pluripotency through Dual Regulation of Signaling and Epigenetic Pathways in Mouse Embryonic Stem Cells. *Cell Stem Cell* **12**, 368–382 (2013).
42. Grabole, N. *et al.* Prdm14 promotes germline fate and naive pluripotency by repressing FGF signalling and DNA methylation. *EMBO Rep.* **14**, 629–637 (2013).
43. Li, J.-Y. *et al.* Synergistic Function of DNA Methyltransferases Dnmt3a and Dnmt3b in the Methylation of Oct4 and Nanog. *Mol. Cell. Biol.* **27**, 8748–8759 (2007).
44. Pawlak, M. & Jaenisch, R. De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state. *Genes Dev.* **25**, 1035–1040 (2011).
45. Dan, J. *et al.* Roles for Tbx3 in regulation of two-cell state and telomere elongation in mouse ES cells. *Sci. Rep.* **3**, 3492 (2013).
46. Palamarchuk, A. *et al.* Tcl1 protein functions as an inhibitor of de novo DNA methylation in B-cell chronic lymphocytic leukemia (CLL). *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2555–2560 (2012).
47. Acampora, D., Giovannantonio, L. G. D. & Simeone, A. Otx2 is an intrinsic determinant of the embryonic stem cell state and is required for transition to a stable epiblast stem cell condition. *Development* **140**, 43–55 (2013).
48. Chen, T., Ueda, Y., Dodge, J. E., Wang, Z. & Li, E. Establishment and Maintenance of Genomic Methylation Patterns in Mouse Embryonic Stem Cells by Dnmt3a and Dnmt3b.

Mol. Cell. Biol. **23**, 5594–5605 (2003).

49. Dawlaty, M. M. *et al.* Loss of Tet Enzymes Compromises Proper Differentiation of Embryonic Stem Cells. *Dev. Cell* **29**, 102–111 (2014).
50. Koh, K. P. *et al.* Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell* **8**, 200–213 (2011).
51. Meissner, A. *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454**, 766–770 (2008).
52. Tahiliani, M. *et al.* Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science* **324**, 930–935 (2009).
53. Tsumura, A. *et al.* Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. *Genes Cells* **11**, 805–814 (2006).
54. Zhou, W. *et al.* HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. *EMBO J.* **31**, 2103–2116 (2012).
55. Bourillot, P. Y. *et al.* Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells* **27**, 1760–1771 (2009).
56. Niwa, H., Ogawa, K., Shimosato, D. & Adachi, K. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460**, 118–122 (2009).
57. Boroviak, T., Loos, R., Bertone, P., Smith, A. & Nichols, J. The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. *Nat. Cell Biol.* **16**, 516–528 (2014).
58. McLaughlin, K. *et al.* DNA Methylation Directs Polycomb-Dependent 3D Genome Re-organization in Naive Pluripotency. *Cell Rep.* **29**, 1974-1985.e6 (2019).
59. Habibi, E. *et al.* Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* **13**, 360–369 (2013).
60. Leitch, H. G. *et al.* Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol.* **20**, 311–316 (2013).

61. von Meyenn, F. *et al.* Impairment of DNA Methylation Maintenance Is the Main Cause of Global Demethylation in Naive Embryonic Stem Cells. *Mol. Cell* **62**, 848–861 (2016).
62. Takeda, K. *et al.* Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci.* **94**, 3801–3804 (1997).
63. Avalle, L. *et al.* STAT3 localizes to the ER, acting as a gatekeeper for ER-mitochondrion Ca2+ fluxes and apoptotic responses. *Cell Death Differ.* **26**, 932–942 (2019).
64. Tang, Y. *et al.* Jak/Stat3 signaling promotes somatic cell reprogramming by epigenetic regulation. *Stem Cells* **30**, 2645–2656 (2012).
65. Wang, L. *et al.* JAK/STAT3 regulated global gene expression dynamics during late-stage reprogramming process. *BMC Genomics* **19**, 183 (2018).
66. Hwang, I.-Y. *et al.* Psat1-Dependent Fluctuations in α-Ketoglutarate Affect the Timing of ESC Differentiation. *Cell Metab.* **24**, 494–501 (2016).
67. Zhang, J. *et al.* LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. *Cell Stem Cell* **19**, 66–80 (2016).
68. Mullen, A. R. *et al.* Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* **481**, 385–388 (2012).
69. Leeb, M., Dietmann, S., Paramor, M., Niwa, H. & Smith, A. Genetic exploration of the exit from self-renewal using haploid embryonic stem cells. *Cell Stem Cell* **14**, 385–393 (2014).
70. Choi, J. *et al.* Prolonged Mek1/2 suppression impairs the developmental potential of embryonic stem cells. *Nature* **548**, 219–223 (2017).
71. Yagi, M. *et al.* Derivation of ground-state female ES cells maintaining gamete-derived DNA methylation. *Nature* **548**, 224–227 (2017).
72. Chen, T., Ueda, Y., Xie, S. & Li, E. A Novel Dnmt3a Isoform Produced from an Alternative Promoter Localizes to Euchromatin and Its Expression Correlates with Activede Novo Methylation. *J. Biol. Chem.* **277**, 38746–38754 (2002).
73. Neri, F. *et al.* Intragenic DNA methylation prevents spurious transcription initiation. *Nature* **543**, 72–77 (2017).

74. Matsuda, T. *et al.* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* **18**, 4261–4269 (1999).
75. Quinlivan, E. P. & Gregory, J. F. DNA digestion to deoxyribonucleoside: A simplified one-step procedure. *Anal. Biochem.* **373**, 383–385 (2008).
76. Pomerantz, S. C. & McCloskey, J. A. [44] Analysis of RNA hydrolyzates by liquid chromatography-mass spectrometry. in *Methods in Enzymology* vol. 193 796–824 (Academic Press, 1990).
77. Neri, F., Incarnato, D., Krepelova, A., Parlato, C. & Oliviero, S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat. Protoc.* **11**, 1191–1205 (2016).
78. Boroviak, T. *et al.* Single cell transcriptome analysis of human, marmoset and mouse embryos reveals common and divergent features of preimplantation development. *Dev. Camb. Engl.* **145**, (2018).
79. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
80. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
81. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010).
82. Lê, S., Josse, J. & Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **25**, 1–18 (2008).
83. Kharchenko, P. V., Silberstein, L. & Scadden, D. T. Bayesian approach to single-cell differential expression analysis. *Nat. Methods* **11**, 740–742 (2014).
84. Gong, T. & Szustakowski, J. D. DeconRNASeq: a statistical framework for deconvolution of heterogeneous tissue samples based on mRNA-Seq data. *Bioinformatics* **29**, 1083–1085 (2013).
85. DNA Global Hypomethylation in EBV-Transformed Interphase Nuclei | Elsevier Enhanced Reader.

https://reader.elsevier.com/reader/sd/pii/S0014482799944348?token=E5105189B8DF052ECFD1BC7C786FEE0739C5C555E2A2F6AF19733D4D3B50F7CBF9B12E8012619A2AC8EFDA25B98A4A93 doi:10.1006/excr.1999.4434.

86. Neri, F. *et al.* Single-Base Resolution Analysis of 5-Formyl and 5-Carboxyl Cytosine Reveals Promoter DNA Methylation Dynamics. *Cell Rep.* **10**, 674–683 (2015).
87. Su, Y.-T. *et al.* Monoubiquitination of Filamin B Regulates Vascular Endothelial Growth Factor-Mediated Trafficking of Histone Deacetylase 7. *Mol. Cell. Biol.* **33**, 1546–1560 (2013).
88. He, J. *et al.* The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. *J. Cell Biol.* **176**, 141–146 (2007).

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