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Quantitative subcellular proteomics using SILAC reveals enhanced metabolic buffering in the pluripotent ground state

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A B S T R A C T

The ground state of pluripotency is defined as a minimal unrestricted epigenetic state as present in the Inner Cell Mass. Mouse embryonic stem cells (ESCs) grown in a defined serum-free medium with two kinase inhibitors (“2i ESCs”) have been postulated to reflect ground-state pluripotency, whereas ESCs grown in the presence of serum (“serum ESCs”) share more similarities with post-implantation epiblast cells. Pluripotency results from an intricate interplay between cytoplasmic, nuclear and chromatin-associated proteins. Here, we perform quantitative subcellular proteomics to gain insight in the molecular mechanisms sustaining the pluripotent states reflected by 2i and serum ESCs. We describe a full SILAC workflow and quality controls for proteomic comparison of 2i and serum ESCs, allowing subcellular proteomics of the cytoplasm, nucleoplasm and chromatin. The obtained quantitative information revealed increased levels of naïve pluripotency factors on the chromatin of 2i ESCs. Surprisingly, the cytoplasmic proteome suggests that 2i and serum ESCs utilize distinct metabolic programs, which include upregulation of free radical buffering by the glutathione pathway in 2i ESCs. Through induction of intracellular radicals, we show that the altered metabolic environment renders 2i ESCs less sensitive to oxidative stress. Altogether, this work provides novel insights into the proteomic landscape underlying ground state pluripotency.

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the Inner Cell Mass (ICM) of the blastocyst. Conventionally, mouse ESCs were derived and maintained in culture media containing serum and leukemia-inhibitory factor (LIF; "serum ESCs"), which maintain pluripotency via JAK/STAT3 and SMAD signaling (Evans and Kaufman, 1981; Martin, 1981). About ten years ago, a defined minimal culture condition for ESC maintenance has been pioneered relying on two small-molecule inhibitors of the mitogen-activated protein kinase (MEK) and glycogen-synthase kinase (GSK3), respectively (“2i ESCs”; Ying et al., 2008). 2i and serum ESCs have been shown to be distinct in their transcriptome and epigenome (Ficz et al., 2013; Habibi et al., 2013; Joshi et al., 2015; Leicht et al., 2013; Marks et al., 2012; von Meyenn et al., 2016; Walter et al., 2016; Weinberger et al., 2016). Due to the high resemblance of 2i ESCs to pre-implantation ICM cells in the embryo, it was postulated that 2i ESCs exist in a distinct state of pluripotency, the so-called “ground state”. On the other hand, the hypermethylated serum ESCs exist in a metastable state that shares features with the more primed early post-implantation embryo (Wray et al., 2010; Ying et al., 2008).

Recent work implicated that, next to the features mentioned above, also the proteome between 2i and serum ESCs is markedly different. For example, it was shown that 2i ESCs are hallmarked by higher levels of core pluripotency factors such as PRDM14, KLF2, KLF4 and NANOG, which is in correspondence to the ‘enhanced’ pluripotency of 2i ESCs (Walter et al., 2016; Weinberger et al., 2016; Yamaji et al., 2013; Yeo et al., 2014). On the other hand, serum ESCs possess higher levels of de novo methyltransferases (DNMTs) and UHRF1, resulting in higher DNA methylation, and increased levels of focal adhesion and cytoskeletal proteins, all signs of early developmental priming (Habibi et al., 2013; Taleahmad et al., 2017). As pluripotency results from an intricate interplay between cytoplasmic, nuclear and chromatin-associated proteins, the subcellular distribution of proteins can greatly affect the pluripotent state. A notable example comprises the transcription factor TFE3, an important regulator for ESRRB. TFE3 localizes in both the cytoplasm and nucleus in 2i ESCs, but is sequestered into the cytoplasm upon 2i withdrawal resulting in developmental priming (Betschinger et al., 2013). Other examples include the Hippo signaling mediators YAP and TAZ, that sustain primed pluripotency when present in the...
cytoplasm, but induce differentiation upon nuclear translocation (Zhou et al., 2017). To further extend our understanding of the molecular mechanisms and pathways that define the pluripotent ground state, quantitative information of the (subcellular) proteomic landscape is critical.

To obtain quantitative proteome information, it is important to minimize variations introduced during sample handling, in particular for subcellular proteomics. Therefore, early mixing of samples during the experimental workflow is highly preferable. One of the most powerful approaches for high-throughput quantitative proteomics that allows for early mixing represents Stable Isotope Labeling with Amino acids in Cell culture (SILAC). SILAC relies on the labeling of proteins with heavy versions of arginine and lysine (the ‘heavy’ sample). Before downstream processing, cell pellets of a heavy labeled sample can be mixed with cell pellets from a sample in which the proteins contain normal arginine and lysine (the ‘light’ sample), after which the mass spectrometer can discriminate between light- and heavy-labeled proteins based on mass. Notably, SILAC labeling approaches for proteomic characterization of 2i and serum ESCs have not been described thus far.

Here, we provide quantitative information on the subcellular proteomes of 2i and serum ESCs using our SILAC approach. Next, we apply our workflow to generate subcellular proteomes of the cytoplasm, nucleoplasm and chromatin-bound fraction of 2i and serum ESCs. Using the information of the proteins as quantified by this approach we present evidence that 2i ESCs are hallmarked by increased nuclear and chromatin levels of naïve transcription factors such as KLF4. Furthermore, the cytoplasmic proteome suggests that the activity of several metabolic pathways between 2i and serum ESCs is changed. Interestingly, these differential pathways render 2i ESCs more resistant to metabolic stress compared to serum ESCs. Altogether, this work provides quantitative insights into the subcellular proteome landscape of the pluripotent ground state.

2. Experimental procedures

2.1. ES Cell culture and SILAC labeling

E14 ESCs (129/Ola background) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 15% fetal bovine serum, 5 μM beta mercaptoethanol (Sigma) and Leukemia inhibitory factor (LIF; 1000 U/ml) (Millipore), referred to as serum ESCs, or in serum-free N2B27 (also called Ndfif) supplemented with PD0325901 (1 μM), CH99021 (3 μM) and LIF (1,000 U/ml), referred to as 2i ESCs. The ESCs have been derived in serum conditions and were adapted to 2i culture. For SILAC labeling, 2i ESCs were grown in customized Ndfif (without Arginine and Lysine; Stem Cell Sciences) supplemented with 13C6,15N4 L-arginine and 13C4,15N2 L-lysine (Isotec). The absence of light arginine and lysine in the SILAC medium was verified via ion exchange chromatography (Fig. S1). Cells were grown in SILAC medium for at least 8 population doublings to ensure full incorporation of the heavy labeled aminocoids. For ROS induction experiments, cells were allowed to attach overnight prior to exposure to the various compounds. H2O2 was used at a range of concentrations and 150 μM for ROS induction. Doxorubicin was used at 500 nM and 1 μM for survival assays and 500 nM for ROS induction. 5-azacytidine was used at 500 nM and 1 μM for survival assays and 1 μM for ROS induction. MG132 was used at 5 μM for both survival and ROS induction assays. For survival assays, inhibitors were added for 20 h (MG132 for 6 h) and for ROS induction ESCs were exposed for 3 h. When DMSO was used as a control, the final DMSO concentration was the same as in compound-treated cells (compounds were dissolved in DMSO and further diluted in PBS).

2.2. Subcellular fractionation

Extracts were prepared as described in Dignam et al. (1983). Cells were harvested with trypsin and washed with PBS. An equal number of heavy-labeled 2i ESCs and non-labeled serum ESCs (grown in regular serum medium) were mixed and pelleted at 400 g for 5 min at 4 °C. The resulting cell pellets were incubated for 10 min at 4 °C in five volumes of Buffer A (10 mM Hapes-KOH (pH 8.0), 1.5 mM MgCl2, 10 mM NaCl) and centrifuged at 400 g for 5 min at 4 °C. Cells were resuspended in two volumes of Buffer A plus protease inhibitors (Roche) and 0.15% NP-40 and transferred to a Dounce homogenizer. After douncing 30–40 strokes with a Type B pestle, the lysates were centrifuged at 3,200 g for 15 min at 4 °C. After centrifugation, the supernatant was taken as the cytoplasmic extract. The nuclear pellet was washed twice with PBS and nuclei were pelleted at 3200 g for 5 min at 4 °C. The nuclear pellet was resuspended in 2 volumes Buffer C (420 mM NaCl, 20 mM Hapes-KOH (pH 8.0), 20% v/v glycerol, 2 mM MgCl2, 0.2 mM EDTA, 0.1% NP-40) supplemented with protease inhibitors and 0.5 mM DTT. This solution was rotated for 1 h at 4 °C and then spun at 20,800 g for 45 min at 4 °C. The supernatant was taken as the nuclear fraction and stored at −80 °C until further use. To obtain the chromatin fraction, the insoluble chromatin pellet was resuspended in four volumes of Radio Immunoprecipitation buffer (RIPA) (150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1% SDS, 50 mM Tris (pH = 8)) and briefly sonicated to solubilize the chromatin fraction and stored at −80 °C until further use.

2.3. RT-qPCR

Total RNA was isolated using a RNasy mini kit (Invitrogen). cDNA was synthesized using random hexamers and RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad).

2.4. Western blot

Protein extracts were denatured in 4× SDS loading dye and separated on a 12% SDS-PAGE gel. Separated proteins were transferred onto a PVDF membrane for staining with primary and secondary antibodies. Primary antibodies used were mouse anti-ACBT (Abcam; #S226), Rabbit anti-Topoisomerase 1 (Abcam; #109374), rabbit anti-HDAC2 (Abcam; #70299) and Rabbit anti-H3K9me3 (Abcam; #8899). Protein bands were visualized using Pierce Western Blotting Substrate (Thermo, #32209) according to the manufacturer’s instructions.

2.5. (Hydroxy)methylation measurements of genomic DNA

Genomic DNA was isolated using the Wizard genomic DNA isolation kit (Promega). Mass spectrometry analysis of the nucleosides was performed on genomic DNA digested using DNA Degradase Plus (Zymo Research). Levels of (hydroxy)methylation were quantified via liquid chromatography-tandem mass spectrometry (LC-MS) as described in Kroese et al., 2014.

2.6. Generation of whole cell extracts

Cell pellets were resuspended in RIPA buffer supplemented with protease inhibitor at a density of 10,000 cells/μl and sonicated for 2 cycles (30 s ON, 30 s OFF) on a Biorupter (Diagenode) to solubilize the cells.

2.7. Mass spectrometry and data analysis

Whole cell, cytoplasmic, nuclear and chromatin extracts were de-natured using Filter Aided Sample Preparation (FASP) and digested using Trypsin/LysC. Trypsin digests were desalted using Stage Tips prior to mass spectrometry measurements. Mass spectra were recorded.
A. Regular 2i vs. SILAC 2i

B. Cumulative cell number vs. passage

C. Percent mC/G vs. passage

D. ΔCt of Oct4, Nanog, Rex1, and Prdm16

E. Number of peptides vs. log2 (peptide ratio H/L)

F. Heavy/Light (log2) replicate 1 vs. replicate 2

G. Relative abundance of peptides

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on an LTQ-Orbitrap QExactive mass spectrometer (Thermo Fisher Scientific) using a 4-h acetonitrile gradient, selecting the top 10 most intense precursor ions for fragmentation. Thermo Raw MS files were analyzed using the MaxQuant software version 1.5.1.0 and searched against the curated UniProtKB mouse proteome (Cox et al., 2014; Cox and Mann, 2008). Default MaxQuant settings were used with the following modifications: multiplicity was set to 2, Arg10 and K8 were selected as heavy labels, match between runs and quantification were enabled. Downstream analysis of proteomics data was performed using Perseus (Tyanova et al., 2016) and in-house R scripts. Differential proteins were calculated using the Significance B feature in Perseus based on the normalized H/L ratios (threshold at p < 0.05) (Cox and Mann, 2008). For an overview of the pathways sustaining 2i and serum ESCs, the main effector proteins were obtained from Hackett and Azim Surani, 2014. The cytoplasmic proteomes were used to obtain cytoplasmic ratios. As fold changes of the pluripotency factors in the nucleus and the chromatin were similar, we used the ratios of the chromatin for the nuclear / chromatin compartment. For a complete overview, the levels of important effector proteins that were not detected in our MS data were inferred from literature.

2.8. Quantification of intracellular ROS and glutathione

ROS levels were quantified using the DCFDA Cellular ROS Detection Assay Kit (Abcam, #ab113851) according the manufacturer’s instructions. Total glutathione levels were quantified using the Glutathione Fluorometric Assay Kit (Biovision, #K264-100) according the manufacturer’s instructions.

2.9. RNAseq analysis

Gene expression counts of preimplantation embryos (E2.5, E3.5, E4.5) and postimplantation embryos (E5.5) were obtained from Boroviak et al., 2015. Counts for the genes (of which the proteins were detected in our SILAC workflow) that are related to ROS scavenging were extracted and Z-score normalized using R3.3.2. Genes with a peak expression (= highest Z-score) at E2.5-E4.5 were considered enriched in the preimplantation stage and those with a peak expression (= highest Z-score) at E5.5 were considered enriched in the postimplantation stage.

2.10. Data accessibility

The proteomic datasets are available from the ProteomeXchange Consortium via PRIDE submission with the dataset identifier PXD009217.

3. Results

3.1. The use of SILAC for whole proteome comparison between 2i and serum ESCs

SILAC-based approaches generally require dialysis of serum to remove the natural arginine and lysine amino acids. During this process, other low-molecular factors including growth factors are removed as well, which might interfere with the cellular identity of sensitive cell types (Xie et al., 2014). To measure the proteomes of 2i and serum ESCs using SILAC, we took advantage of the serum-free growth conditions of 2i ESCs. For the SILAC experiments, we cultured 2i ESCs in custom defined medium lacking regular arginine and lysine (Fig. S1) but supplemented with heavy lysine and arginine. The “heavy” labeling of 2i ESCs allows for a SILAC-based comparison to serum ESCs that are grown in normal medium (i.e. ‘light’ labeled). First, we evaluated whether culturing in SILAC medium did not result in unanticipated changes to the 2i ESCs. During culturing for multiple passages, the 2i ESCs retained their typical round morphology (Fig. 1A) (Ying et al., 2008). Also, the growth rate of 2i ESCs in normal medium or SILAC medium supplemented with either ‘light’ (‘normal’) or ‘heavy’ amino acids was similar (Fig. 1B). In light of the fact that global DNA hypomethylation is a major hallmark of 2i ESCs (Habibi et al., 2013), we measured the DNA methylation levels of SILAC 2i ESCs using mass spectrometry. This revealed that 2i ESCs in both normal medium and SILAC medium exhibit a similar degree of DNA hypomethylation (Fig. 1C). Finally, we measured the expression of key pluripotency factors Oct4, Nanog, Rex1 and the stem cell maintenance gene Prdm16 using RT-qPCR. This showed that the expression of these genes is not affected by the SILAC medium formulation (Fig. 1D). Altogether, this indicates that the SILAC medium does not largely affect the pluripotent state of 2i ESCs.

Next, we evaluated the efficiency of labeling with heavy amino acids of 2i ESCs grown in SILAC medium (Fig. 1E). We performed a single LC-MS experiment of 2i ESCs grown in heavy medium for six passages and inspected the heavy/light ratios in this sample. This led to a unimodal distribution of high incorporation ratios of heavy amino acids (incorporation rate > 97%), demonstrating that 2i ESCs can be efficiently labeled. We also queried the spectra for heavy proline in light of recent work reporting conversion of heavy arginine to heavy proline (Van Hoof et al., 2007). However, at the arginine concentration used for the current study we did not detect any significant conversion of heavy arginine into heavy proline (data not shown). Finally, to test the reproducibility of the workflow, we generated SILAC proteomes of two independent experiments. This allowed quantification of over 4000 proteins with high reproducibility (R = 0.88) between the experiments, as well as the identification of differential proteins (Fig. 1F,G). Altogether, we concluded that our SILAC workflow is robust and applicable for quantitative proteomics of 2i and serum ESCs.

3.2. Subcellular proteomes of 2i and serum ESCs using SILAC

An important advantage of SILAC over other MS approaches such as label-free quantification (LFQ) or dimethyl labeling is the possibility of early mixing of different cell populations, which minimizes variations introduced by sample preparation prior to LC-MS analysis (Lau et al., 2014). As such, SILAC is highly suitable for LC-MS experiments that require subcellular fractionation. To obtain subcellular information of 2i and serum ESCs, we separated the cytoplasm, nuclei and chromatin of SILAC-labeled 2i ESCs and serum ESCs (Fig. 2A). We validated proper separation of the fractions using western blot for known protein markers that are mainly cytoplasmic b-actin, nuclear (Topoisomerase I), nuclear and chromatin-associated (HDAC2) and chromatin only
**A**

Diagrams showing the distribution of proteins in different cell compartments:

- Cytoplasm (n=2295)
- Nucleoplasm (n=2298)
- Chromatin (n=1841)

**B**

Heatmaps illustrating differential protein expression:

- Differential cytoplasm (158)
- Differential nucleoplasm (192)
- Differential chromatin (131)

**C**

Bar charts comparing replicate 1 and replicate 2 for various proteins:

- ACTB
- Topo I
- HDAC2
- H3K9me3

**D**

Venn diagrams showing detected proteins in cytoplasm, nucleus, and chromatin:

- Detected proteins
- Up 2i
- Up Serum

**E**

Venn diagrams depicting the overlap of detected proteins in different compartments:

- Cytoplasm
- Nucleus
- Chromatin

**F**

Venn diagrams showing the overlap of proteins in cytoplasm and nucleus for 2i and Serum conditions:

- Up 2i
- Up Serum

*(caption on next page)*
D) Scatter plot of fold changes heavy / light in the subcellular fractions of two independent SILAC experiments. Pearson correlation between the two replicates is indicated in left top. Blue dots are proteins significantly higher in serum ESCs in both replicates and green dots are proteins significantly higher in 2i ESCs (p < 0.05; significance B). Differential proteins of interest have been highlighted. Bottom: Numbers and GO terms belonging to the differential proteins (p < 0.05 using significance B in both replicates) in the subcellular fractions. E) Venn diagram representing the overlap between identified proteins (at least 2 peptides) in the various fractions. F) Venn diagrams representing the overlap between differential proteins in the various fractions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(H3K9me3) (Fig. 2B). After mass spectrometry, gene ontology (GO) analysis of the proteins as identified within each of the fractions revealed GO-terms relevant to the specific subcellular fractions: these included mainly metabolic terms for the cytoplasmatic fraction, RNA-splicing and transport for the nuclear fraction and chromatin-modification and gene expression for the chromatin fraction (Fig. 2C). Taken together, this demonstrates that the subcellular fractions were adequately separated.

For all three replicates, we identified up to 2300 proteins per fraction with high reproducibility (Fig. 2D, E). Focusing on differential proteins (p < 0.05 in both replicates), 158 proteins were significantly different in both replicates between 2i and serum ESCs in the cytoplasmatic fraction, 192 in the nuclear fraction and 131 in the chromatin fraction (Fig. 2D; Supplementary Table 1). Notably, we observed limited overlap in detected and differential proteins between the various fractions (Fig. 2E, F), further highlighting the importance of subcellular fractionation. The differential proteins that we identify included notable markers for ground state pluripotency such as DAZL, ESRRB and TFCP2L1 enriched in 2i ESCs. Proteins significantly enriched in serum ESCs include the DNA methylation associated DNMT3A/B/L and UHRF1, which is in line with the higher DNA methylation levels in serum ESCs, as well as the early-differentiation associated proteins LIN28A and UTF1, which is in agreement with the metastable state of serum ESCs (Fig. 2D, Supplementary Table 1) (Galonska et al., 2014; Zhang et al., 2016). Also GO terms related to the differential proteins included priming-related terms for serum ESCs such as embryo development and neural tube closure in the nucleoplasm and DNA methylation for the chromatin-associated proteins (Fig. 2D). We also evaluated the levels of the factors TFE3 and YAP1 (as mentioned in the introduction). TFE3 was present in the cytoplasm and nucleus of 2i and serum ESCs, in line with previous reports (Supplementary Table 1). We also observed YAP in all fractions in 2i and serum ESCs, which seems to be tolerated by the undifferentiated ESCs. In summary, these results show that the SILAC workflow is suitable to obtain subcellular proteomes of 2i and serum ESCs with high resolution.

3.3. SILAC identifies pluripotency modules enriched in ground state pluripotency

The quantitative information obtained on the subcellular proteome of 2i and serum ESCs provides the unique opportunity to investigate how the differentially activated pathways in the cytoplasm affect the abundance of individual members of the core pluripotency network on the chromatin. First, we inspected the cytoplasmic levels of the direct targets of extracellular signaling cues, which comprise SMAD1/5/8 and STAT3 in serum ESCs and GSK3, STAT3 and ERK1/2 in 2i ESCs (Hackett and Azim Surani, 2014). The total abundance of these proteins is not different between 2i and serum ESCs (Fig. 3), which is in line with previous reports showing the state of phosphorylation and/or the capacity to phosphorylate is affected rather than the abundance of these proteins (Ying et al., 2008). Next, we focused on the abundance of the core pluripotency network on the chromatin making use of a comprehensive list of pluripotency factors (Dunn et al., 2014). This revealed that the levels of OCT4 and SOX2 are equal between 2i and serum ESCs, which fits with their fundamental importance for the maintenance of pluripotency (Masui et al., 2007). The pluripotency factor REST was also similarly present on the chromatin in 2i and serum ESCs. We observed that, although LIF is present in the culture conditions of both 2i and serum ESCs, the main downstream effectors KLF4 and TFCP2L1 were strongly enriched in 2i ESCs, suggesting their expression level is affected by factors other than LIF (Fig. 3). Further, we noted 2i ESCs are hallmarkmed by increased levels of the ESRRB, which is the key target of b-catenin (Martello et al., 2012), TBX3 and NANOG. Individually these factors are dispensable for the maintenance of pluripotency, but they strengthen the pluripotency network (Dunn et al., 2014). Hence, the increased chromatin occupancy of these factors is in line with previous observations on the pluripotent state of 2i ESCs (Wray et al., 2010). Interestingly, we observed that the ancillary factors KLF5 and SALL4 are enriched on the chromatin in serum ESCs (Fig. 3). Removal of KLF5 and SALL4 results in differentiation of serum ESCs, whereas at least SALL4 ablation is well tolerated by 2i ESCs. This suggests that these factors become more important upon dissolution of the pluripotent state (Ema et al., 2008; Miller et al., 2016). Altogether, these analyses reveal that the pluripotency network is plastic and changes between pluripotent states.

3.4. Ground state and metastable pluripotency are supported by distinct metabolic programs

Next to epigenetic changes, changes on the metabolic level are starting to be uncovered between 2i and serum ESCs (Carey et al., 2015). Subcellular fractionation brings lower abundant metabolic proteins into the dynamic range of the mass spectrometer, allowing to comprehensively profile proteins belonging to metabolic pathways (Paulo et al., 2013). To obtain a detailed overview of the metabolic pathways that distinguish 2i and serum ESCs, we analyzed the differential proteins in the cytoplasmatic fraction for metabolic gene ontology (GO) terms using the Metaanalyst software (Xia et al., 2015). This revealed that GO terms characterizing 2i ESCs were mainly related to fatty acid and sugar metabolism, whereas GO-terms related to serum ESCs included acetyl-CoA biosynthesis and glycolysis (Fig. 4A). The enrichment of glycolysis factors in serum ESCs, as exemplified by the upregulation of key factors PDK1, LDHA and ZIC3, fits with their metastable state as the enhancement of glycolysis is a hallmark for induction of primed pluripotency (Fig. 4B) (Zhou et al., 2012). Interestingly, we observed that one of the top enriched terms in 2i ESCs is glutathione metabolism (Fig. 4A; indicated in red). Together with Catalase (Cat), Peroxiredoxins (PRX) and Thioredoxins (TRX), the Glutathione system is of critical importance for reducing oxidative stress by scavenging free radicals such as H2O2. This is established through glutathione (GSH), which is oxidized by free radicals towards glutathione disulfide (GSSG) that can be actively reduced to GSH (Fig. 4C) (Filomeni et al., 2002). Most of the scavenging proteins involved in glutathione metabolism as well as catalase are significantly upregulated in 2i ESCs (Fig. 4C). To investigate whether the upregulated glutathione pathway proteins are also reflected in the amount of glutathione, we measured the total levels of the glutathione (the sum of GSH and GSSG). This was similar between 2i and serum ESCs (Fig. 4D). As the glutathione pathway is involved in ROS scavenging and 2i ESCs are characterized by upregulation of metabolic programs, this could result in increased reactive oxygen species (ROS) levels as these are by-products of metabolic processes (Ray et al., 2012). Therefore we
measured intracellular ROS levels, which revealed significant higher levels of ROS in 2i ESCs as compared to serum ESCs (Fig. 4E). As free radicals can attack peptide backbones resulting in oxidized proteins, this could implicate a higher presence of oxidized proteins in 2i ESCs. To evaluate this, we used the dependent peptide search in Maxquant to this could implicate a higher presence of oxidized proteins in 2i ESCs.

To further investigate these findings, we assayed whether the increased glutathione pathway proteins would reflect more efficient ROS buffering in 2i ESCs. To this end, we treated 2i and serum ESCs with the chemical compounds doxorubicin (induction of DNA damage), 5-azacytidine (induction of DNA hypomethylation) and MG132 (a proteasome inhibitor), which all can induce intracellular ROS (Alexandrova et al., 2008; Friesen et al., 1999; Gao et al., 2008). After exposure to these compounds, we observed that the survival rate of 2i ESCs was higher than that of serum ESCs. In addition, doxorubicin and 5-azacytidine induced more ROS in serum ESCs as compared to 2i ESCs (Fig. 4G-H, S2A-B), which further suggests that 2i ESCs are more efficient in eliminating ROS. Notably, although 2i ESCs showed better survival to proteasome inhibition, this treatment did not induce ROS (Fig. S2B), suggesting that this might be mediated through other mechanisms. To further substantiate the enhanced buffering capacity of 2i ESCs, we added the same concentration of the radical H2O2 to the culture medium of 2i and serum ESCs. This resulted in significantly more intracellular ROS in serum ESCs compared to 2i ESCs, further demonstrating the enhanced metabolic buffering in 2i ESCs (Fig. 4I). Unexpectedly, we observed a slightly compromised survival of 2i ESCs to the addition of exogenous H2O2 as compared to serum ESCs (Fig. S2C). As intracellular ROS levels in treated 2i ESCs were only marginally higher compared to control cells, this is likely the result of interference of H2O2 with other processes or induction of damage to important cellular constituents such as the DNA. Altogether, these assays reveal that 2i ESCs are more efficient at buffering ROS.

The conversion of 2i to serum ESCs represents a powerful model for in vivo development (Marks and Stunnenberg, 2014). Therefore, we investigated whether the increased abundance of the proteins comprising the glutathione pathway is also observed in vivo. To this end, we analyzed RNA-seq data generated from morula (E2.5), the Inner Cell Mass (ICM, E3.5), preimplantation epiblast (E4.5) and post implantation epiblast (E5.5) (Boroviak et al., 2014). We observed that the expression of most subunits of the glutathione pathway was enriched in vivo development (Winkler et al., 2011; Yant et al., 2003). On the other hand, the
4. Discussion

SILAC-based workflows have been rapidly adopted in quantitative proteomics because of the robustness and simplicity. A major advantage of SILAC over label free proteomics (LFQ) or chemical labeling strategies such as dimethyl labeling is that it allows for early mixing of samples. This results in minimization of variation introduced during sample preparation and enhanced precision and reproducibility of quantification (Lau et al., 2014). As such, SILAC is typically suited for subcellular proteomics, which allows both studying molecular pathways as well as identification of low-abundant proteins (e.g. pluripotency and other transcription factors) through reduction of sample complexity, which brings these proteins into the dynamic range of the mass spectrometer (Graumann et al., 2008). Here, we show that SILAC can be adopted for the subcellular comparison of 2i and serum ESCs. This is particularly powerful with the use of 2i ESCs as these are grown in chemically defined medium in which normal ("light") lysine and arginine can conveniently be substituted by “heavy” lysine and arginine. Here, we mixed 2i and serum ESCs prior to subcellular fractionation, which minimizes variations introduced due to sample handling. We observed a slight skewing of the non-normalized heavy/light ratios towards the light channel, which is resolved upon normalization.

For future applications of the SILAC workflow, normalization of the mixing on cellular level and protein level might be beneficial to determine the optimal experimental workflow.

Significant efforts have been made to disentangle the proteome of 2i and serum ESCs (Taleahmad et al., 2015, 2017). Here we complement these studies using SILAC-based quantitative subcellular proteomics, which improves on the quantification accuracy and simultaneously increases the depth of the proteome. This allowed us to provide quantitative insights into the subcellular proteome of 2i and serum ESCs. Among others, we investigated the pluripotency modules sustaining 2i and serum ESCs. Previous reports demonstrated that 2i ESCs are characterized by the upregulation of naïve pluripotency factors (Dunn et al., 2014; Mzoughi et al., 2017; Qiu et al., 2015). Here, we provide evidence that upregulation of these naïve factors in 2i ESCs is accompanied by enrichment of these factors on chromatin. As such, a comprehensive analysis of genomic binding sites of naïve factors such as TCFP2L1 and TBX3 in 2i and serum ESCs might provide further insights into the maintenance of the ground state of pluripotency. Next to factors that characterize 2i ESCs, we identify pluripotency factors that are enriched in serum ESCs, such as SAL14 and KLFS. It has been shown that Sal14 and Klfs mutant ESCs grown in serum show strong signs of premature differentiation. Therefore, it would be interesting to determine the genomic locations of these factors in 2i and serum ESCs, as well as in early differentiated cell types, to pinpoint the role of these factors in pluripotency and the exit thereof.

The changes as we observe in the pluripotency network between 2i and serum ESCs are likely to affect the epigenetic state, as epigenetic changes have been directly linked to relocalization of core pluripotency factors (Galonska et al., 2015). In addition to the epigenetic environment, the pluripotency circuit has been found to orchestrate the metabolic state of pluripotent stem cells. For example, ESRRB was shown to enhance oxidative phosphorylation and is therefore essential for reprogramming of somatic cells to naïve pluripotency. ZIC3 promotes reprogramming towards primed pluripotency by enhancing glycolysis (Lim et al., 2007; Sone et al., 2017). The core pluripotency factor OCT4 was found to be directly involved in regulation of glycolysis and the serum ESC specific factor MYC (Marks et al., 2012) has been linked to the glutamine-dependence of serum ESCs (Carey et al., 2015; Wise et al., 2008). Also LIN28A/B, direct targets of FGF signaling, have been shown to modulate metabolic pathways towards glycolysis to facilitate priming of ESCs (Zhang et al., 2016). Previous analyses on the RNA level suggested that there might be differences in metabolism between 2i and serum ESCs (Marks et al., 2012). Here we extend these analyses with quantitative information on the cytoplasmic proteome. Our analyses of the members of metabolic pathways indicated that serum ESCs are enriched for glycolysis. However, previous observations revealed similar use of the glycolysis pathway in 2i and serum ESCs (Carey et al., 2015). As such, this indicates that serum ESCs are inducing transcriptional networks that are required to fully switch to glycolysis metabolism as characteristic for the in vivo post implantation epiblast as well as epiblast-derived stem cells (EpiSCs) (Zhou et al., 2012). This further supports the observation that serum ESCs represent a more primed version of pluripotency compared to 2i ESCs and suggests that the enhancement of glycolysis is readily initiated upon dissolution of the pluripotent ground state.

We show that 2i ESCs contain increased levels of proteins involved in the glutathione pathway, as well as enhanced resistance to intracellular ROS generation as compared to serum ESCs. As total levels of glutathione itself are similar between 2i and serum ESCs, this suggests that the turnover of oxidized and reduced glutathione, during which ROS are scavenged, is more efficient in 2i ESCs. This might be required as 2i ESCs are metabolically more active and hence are likely to generate more free radicals during metabolic processes such as oxidative phosphorylation as stimulated by ESRRB. Whether this is also relevant for the preimplantation embryo remains to be investigated, although our RNA-seq analysis indicates that the glutathione pathway is mostly active before implantation. Interestingly, the formation of ROS, and consequently the enhanced buffering, have been shown to affect the levels of DNA methylation (Wu and Ni, 2015). Given that 2i ESCs and preimplantation embryos are hallmarked by global DNA hypomethylation, it might well be that the enhanced glutathione metabolism in ground state pluripotency contributes to epigenetic features such as DNA hypomethylation.
5. Concluding remarks

In this work we present a SILAC workflow to reliably generate subcellular proteomes of pluripotent states represented by mouse embryonic stem cells (ESCs) maintained in distinct culture media. Using SILAC, we show that the pluripotent ground state as presented by 2i ESCs is hallmarked by distinct metabolic programs as compared to more primed serum ESCs. Future studies using in-depth metabolomics profiling will be required to further investigate the metabolic programs as identified here and how these contribute to ground state pluripotency. Moreover, the application of this SILAC workflow can be used to comprehensively study the molecular pathways sustaining pluripotency, for example through combining subcellular fractionation with phospho- or ubiquitin-proteomics, which could uncover novel signaling cascades. Furthermore, SILAC could be applied in proteomic comparisons of 2i and serum ESCs for which early mixing of cells is highly beneficial, such as organelle enrichment and interactome studies (Li et al., 2012).

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Conflicts of interest

The authors have declared no conflict of interest.

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