Pluripotency can be captured in vitro in the form of Embryonic Stem Cells (ESCs). These ESCs can be either maintained in the unrestricted “naïve” state of pluripotency, adapted to developmentally more constrained “primed” pluripotency or differentiated towards each of the three germ layers. Epigenetic protein complexes and transcription factors have been shown to specify and instruct transitions from ESCs to distinct cell states. In this study, proteomic profiling of the chromatin landscape by chromatin enrichment for proteomics (ChEP) is used in mouse naïve pluripotent ESCs, primed pluripotent Epiblast stem cells (EpiSCs), and cells in early stages of differentiation. A comprehensive overview of epigenetic protein complexes associated with the chromatin is provided and proteins associated with the maintenance and loss of pluripotency are identified. The data reveal major compositional alterations of epigenetic complexes during priming and differentiation of naïve pluripotent ESCs. These results contribute to the understanding of ESC differentiation and provide a framework for future studies of lineage commitment of ESCs.

Pluripotency is a transient state during embryonic development in which cells in the epiblast are capable of forming all somatic cell types and germ cells.[11] After implantation in the uterus, pluripotent cells progressively gain restrictive epigenetic features that constrain their developmental potential.[12] This resulted in the dogma that there is no single pluripotent state, but rather a spectrum of pluripotent states ranging from “naïve” to “primed,” with the major discriminator being that primed Embryonic Stem Cells (ESCs) are receptive to differentiation-inducing cues from the environment.[13,14] Several distinct populations of pluripotent cells can be maintained in vitro, each reflecting a particular time point in embryonic development.[15] Naïve ESCs of mice can be derived from pre-implantations embryos and can be maintained in fetal calf serum supplemented with Leukemia Inhibitory Factor (LIF),[5,6] which mediate activation of the SMAD and JAK-STAT signaling, respectively.[2] Upon injection into pre-implantation embryos, these cells can contribute to all germ layers and the germline.[3] The in vitro cultured equivalents of post-implantation pluripotent cells are Epiblast stem cells (EpiSCs), of which the maintenance relies on stimulation of SMAD and ERK signaling by Activin A and FGF2, respectively.[7,8] EpiSCs are more developmentally constrained and represent the “primed” state of pluripotency, as EpiSCs are not germline-competent and do not contribute efficiently to embryos in chimera assays.[9,10] However, EpiSCs retain the ability to form teratomas, demonstrating their pluripotency,[8] although EpiSCs cannot revert to the naïve state of pluripotency. When primed ESCs progress with dissolution of the pluripotent state, they initiate lineage specification programs during differentiation and as such are no longer considered pluripotent.[11]

The transition from naïve to primed and subsequently differentiation requires extensive rewiring of the cellular state, exemplified by major changes in cell morphology and distinct metabolic, transcriptional and epigenetic states.[15] Some of these changes, such as the activation of lineage-priming genes and increase of DNA methylation, are readily initiated in primed pluripotency.[11,14] Another notable example comprises bivalent chromatin domains, which are decorated with both repressive and activating histone marks,[13] that are largely resolved when the pluripotent state is lost, starting upon priming of ESCs.[16]

Epigenetic processes play a substantial role in regulation of cell fate decisions. The effector proteins on the chromatin, Transcription Factors (TFs), often exhibit a role as “master regulator” by binding to and regulating many genes, thereby driving cell state transitions.[17] Despite their low abundance, transcription factors make up a significant portion of the variation in the mammalian proteome,[18] and exert critical roles in mammalian development.[17] Several regulatory TFs have been discovered that drive early priming or differentiation in the embryo, such as Otx2.
and Zeb1/2, respectively.\[^{19}\] In addition, recent reports have highlighted dramatic rewiring of epigenetic complexes between ESCs and early differentiated cell types such as neural progenitor cells (NPCs).\[^{20,21}\] However, a comprehensive overview of the chromatin environment during priming and differentiation is currently lacking. Such an overview would provide valuable information on TFs and other epigenetic factors in the process of differentiation. Here, we set out to provide a comprehensive overview of the chromatin proteome during the onset of differentiation.

As epigenetic factors and TFs are generally lowly abundant,\[^{18}\] their detection is challenging when using proteomics approaches that profile total cell lysates.\[^{22}\] However, these factors can be brought into the dynamic range of mass spectrometer through enrichment of the chromatin fraction.\[^{21-26}\] Simultaneously, this provides information on the levels of these factors in their relevant context.\[^{27,28}\] To gain insight in dynamics of epigenetic regulators and transcription factors during pluripotency and differentiation, we collected a range of cell types representing naïve pluripotency ("ESCs"), primed pluripotency ("EpiSCs"), and early neuronal differentiation ("END"), which is a widely adopted differentiation system.\[^{29}\] (Figure 1A). First, we confirmed that the various cell types were morphologically distinct. In particular, ESCs formed small colonies, EpiSCs formed large, flatter colonies, and END cells were hallmarked by a more stretched morphology.\[^{30}\] (Figure 1B). In terms of expression of known marker genes, the naïve pluripotency marker Rex1 was highly expressed in ESCs, but not in other cell types. EpiSC culture conditions showed high expression of EpiSC markers Otx2, Fgf5, and Zic2.\[^{31}\] Ectodermal differentiation markers Pax6 and Sox1 were upregulated in END cells (Figure 1C).\[^{31}\] These results validated our in vitro differentiation protocol.

Next, we aimed to isolate the chromatin of these cells using Chromatin Enrichment for Proteomics (ChEP)\[^{23}\] (Figure 2A). To confirm that ChEP enriches for chromatin, we validated the enrichment for histones after ChEP of ESCs as compared to whole cell extracts of ESCs using Coomassie staining (Figure S1A, Supporting Information). Next, we generated chromatin proteomes of ESCs and compared these to whole cell proteomes of ESCs. We observed that the ChEP strongly enriches for chromatin factors such as histones and DNA binding zinc finger proteins, while ChEP depletes for cytosolic factors such as mitochondria and translation initiation factors (Figure S1B-D), validating the ChEP procedure. Next, we performed ChEP for ESCs, EpiSCs, and END cells and performed mass spectrometry analyses of the chromatin fraction. A total of 4174 proteins were reproducibly quantified (Figure S1E, Supporting Information) and the replicates showed a high correlation (spearman correlation >0.95) (Figure 2B,C). Next, as chromatin enrichment procedures can be prone to contamination from organelles such as the mitochondria,\[^{32}\] we assessed the purity of the ChEP proteomes on the peptide level. This revealed that of all detected proteins, 70% (ESCs), 60% (EpiSCs), and 63% (END cells) of the unique and razor peptides originated from proteins with an expected chromatin function.\[^{33}\] For further downstream analysis, only the proteins with an expected chromatin function (Experimental Section; Table S2, Supporting Information) were included.

For validation of the cell types on the chromatin level, we plotted the Label Free Quantification (LFQ) values of known markers of EpiSCs and early differentiation, which included naïve markers (TBX3 and KLF4), priming markers (DNMT3B and GRHL2), and neuronal markers (FOXP1 and HMGN3).
Figure 2. ESCs, EpiSCs and END cells show distinct chromatin proteome signatures. A) Schematic overview of workflow that was applied for ChEP-MS. B) Spearman correlation of all ChEP samples. C) Scatter plot of two replicas of ESC chromatin proteomes. Spearman correlation is indicated in the plot. D) log2 LFQ values for proteins associated with different cell states: TBX3 and KLF4 with naïve pluripotency, DNMT3B and GRHL2 with primed pluripotency, and FOXP1 and HMGN3 with neuronal development. E) Heatmap showing all differential proteins (ANOVA, BH-corrected FDR < 0.05) clustered on z-score. Number of proteins per cluster is marked, relevant GO terms associated with each cluster are mentioned.
Next, we focused on DNA methylation as this is a major driver of differentiation. \[39\] We observed higher levels of DNMT3B in EpiSCs, in line with previous reports,\[40\] but an increase in DNMT3A in END cells (Figure 3A), which could indicate a switch in DNMT3 proteins during initiation of differentiation. We also observed a drastic downregulation TET1/2 in both EpiSCs and END cells, which fits previous reports showing the downregulation of TET1/2 during embryoid body differentiation.\[41\] In addition, this further indicates that the altered DNA methylation landscape in differentiating cells may be the result of a shift in TET1/2 and DNMT3A/B balance, rather than a unilateral increase in depositing enzymes.\[39,42\]

The last category of proteins we focused on is pluripotency factors as these comprise dynamic regulators of cell fate and differentiation. We observed that both EpiSCs and END cells displayed an overall downregulation of pluripotency markers, fitting the loss of the naive state (Figure S1H, Supporting Information). In addition, EpiSCs were characterized by increased levels of LIN28A/B which is characteristic for primed pluripotency\[43\] (Figure S1H). Collectively, our analysis reveals strong rewiring of chromatin-associated epigenetic complexes upon induction of differentiation.

Finally, we aimed to use our chromatin proteomes to identify candidate TFs that regulate differentiation. We filtered the proteins identified in the chromatin proteomes for TF activity using a combination of published TF databases\[44,45\] and identified differential TFs (\(p < 0.05\) and >twofold difference) relative to ESCs. These analyses identified known naïve pluripotency factors such as ESRRB and KLF2 to be more abundant in ESCs (Figure 3B). Conversely, factors associated with neural development such as FOXC1/2 and ZEB1 are enriched in END cells,\[46,47\] and priming factors such as GRHL2 and LIN28B in EpiSCs.\[43,48\] Next to identification of known regulators, we identify several TFs such as SMAD2, ZFHX3, HIC1, and ZHX2 that could be candidate regulators for priming or differentiation.

Here, we provide a comprehensive overview of the chromatin during transition from the naïve pluripotent state toward primed and differentiating cell-states. Focusing on the chromatin proteome enabled detection of low abundant transcription factors, which allowed us to effectively explore these regulatory factors during maintenance and exit of pluripotency. Several of the changes in the chromatin protein landscape during differentiation as observed in the current study are complementary to previous work,\[34\] in which we compared two different states of naïve pluripotency, represented by 2i ESCs and serum ESCs. An example comprises PRC2, which is moderately downregulated in the transition from 2i to serum ESCs, and more drastically upon differentiation. This suggests that epigenetic changes that are linked to differentiation are readily initiated upon dissolution of the pluripotent ground state.

In conclusion, the dataset as generated in the current study recapitulates known dynamics in epigenetic protein complexes during differentiation and can be used to identify novel candidate proteins for future studies. To facilitate this, we have included an extensive table with the proteins identified in this study, their abundance, and whether they are significantly different between ESCs, EpiSCs, and END cells. In addition, we have highlighted whether a protein is considered a chromatin factor (Table S2, Supporting Information). Overall, these data provide a useful resource for future studies on the chromatin environment during maintenance and exit of pluripotency.
Figure 3. Rewiring of epigenetic complexes. A) Log2 fold change (relative to ESCs) of epigenetic complexes and DNA methylation associated factors in both EpiSCs and END cells. B) Candidate regulators of priming and differentiation. Proteins of interest with $p < 0.05$ (ANOVA) and $\geq$ twofold differential have been highlighted. Further details on significant proteins not labelled by name are present in Table S2, Supporting Information.
Experimental Section

Cell Culture and Differentiation: Mouse ESCs were obtained from The Global Bioresource Center ATCC (www.atcc.org) and cultured in DMEM supplemented with 15% Fetal Bovine serum (HyClone), 1000 U mL⁻¹ β-mercaptoethanol and Sodium Pyruvate. Cells were passaged every 2–3 days using Trypsin-EDTA (Gibco, cat. no. 25300120). Mycoplasma was confirmed to be absent in the cell cultures. Epiblast Stem Cells were generated by culturing ESCs in NDiFF medium supplemented with 5 ng/mL FGF5 (Peprotech, cat. no. 100–18B) and 8 ng mL⁻¹ Activin A (R&D Systems, cat. no. 338-AC) for 5 days, after which cells were passaged in clumps every 2–3 days using collagenase type II (Worthington, cat. no. LS004176).[49] Neuro-ectoderm differentiation was induced by adapting serum-grown ESCs to NDiFF without any further supplements for at least 7 days. Cells were split 1:1 using Accutase (Gibco, cat. no. A11105-01).

Chromatin Enrichment for Proteomics: Chromatin was harvested and enriched as described in Kustatscher et al.[23] Cells were cross-linked on plates using 1% formaldehyde and incubated at 37 °C for 10 min. Cross-linking reaction was stopped by adding glycine to a concentration of 0.25 M for 5 min. Plates were rinsed with Phosphate Buffered Saline (PBS) and scraped in 5–10 mL of PBS into tubes. Tubes were centrifuged at 1000 g for 3 min, supernatant was aspirated and cells were resuspended and homogenized in 1 mL cold lysis buffer (25 mM TRIS pH 7.4, 0.1% Triton X-100, 85 mM KCl, 1X Roche protease inhibitor). Suspensions were centrifuged at 2300 g for 5 min at 4 °C, supernatant was aspirated and pellets were resuspended in 500 µL of lysis buffer and incubated at 37 °C for 15 min. Suspensions were centrifuged at 2300 g for 10 min at 4 °C, supernatant was aspirated; pellets were resuspended in 500 µL of SDS buffer (10 mM TRIS pH 7.4, 10 mM EDTA, 4% SDS, 1X Roche protease inhibitor) and incubated at room temperature for 10 min. 1.5 mL of urea buffer (10 mM TRIS pH 7.4, 1 mM EDTA, 8 m urea) was added with samples and they were centrifuged at 16 100 g for 30 min at room temperature. Supernatant was aspirated and pellets were resuspended in 500 µL SDS buffer, after which 1.5 mL urea buffer was added and suspensions were centrifuged at 16 100 g for 25 min at room temperature. Supernatant was discarded and pellets were carefully resuspended in 100–200 µL of storage buffer (10 mM TRIS pH 7.4, 1 mM EDTA, 25 mM NaCl, 10% glycerol, 1X Roche protease inhibitor) and sonicated for 6 min at high intensity (30 s on/off alternation) on a NGS bioruptor (Diagenode). Protein concentration was determined using a Qubit assay (Invitrogen). Samples were subjected to mass spectrometry sample preparation or western blot.

Western Blot: ChEP samples were diluted in 4X SDS buffer (10% SDS, 10 mM β-mercaptoethanol, 20% glycerol, 200 µM TRIS-HCl pH 6.8, 0.05% bromphenolblue) and incubated at 95 °C for 30 min to reverse cross-linking. Samples were loaded onto an 8% or 12% acrylamide separating gel (3.2 mL H₂O, 4 mL acrylamide, 2.6 mL 1.5 mM TRIS pH 8.8, 200 µL 10% SDS, 200 µL 10% ammonium persulfate (AP), 20 µL TEMED) topped with stacking gel (2.975 mL H₂O, 1.25 mL 0.5 M TRIS-HCl pH 6.8, 50 µL 10% acrylamide, 50 µL 10% AP, 5 µL TEMED). This was blotted on Polyvinylidene difluoride (PVDF) membranes and blocked for 1 h in 5% milk in TBST. Membranes were then incubated in 2.5% milk in TBST containing primary antibody o/n at 4 °C. Primary antibodies used are anti-ZMYND8 (Atlas, Cat# HPA020949), anti-H3 (Abcam, Cat# 1791), and anti-EED (Millipore, Cat# 09–774). After washing with TBST, membranes were incubated in 2.5% milk in TBST containing Horse Radish Peroxidase (HRP) conjugated secondary antibody (for rabbit: Dako, cat. no. P0161; for mouse: Dako, cat. no P0217) for 1 h at room temperature. Antibodies were visualized via chemiluminescence (SuperSignal West Pico Plus, Thermo Fisher).

RT-qPCR: Cell pellets were generated by taking a small volume of cells in suspension and centrifuging samples for 3 min at 1000 g. RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized by reverse transcription as previously described.[30] Quantitative PCR on the cDNA was performed using SYBR Green (Bio-Rad, cat. no 1708886). Primers are listed in Table S1, Supporting Information.

Mass Spectrometry Sample Preparation: Sample preparation for mass spectrometry was adapted from published methods.[31] All centrifugation steps were performed at 20 °C unless specified otherwise. Cross-linked samples were incubated at 95 °C for 30 min in decrosslink buffer (10.5 µM Tris-HCl pH 8.5, 19.5% SDS, 60 mM acetic acid), and then incubated at 37 °C for 1 h. After incubation, 5% acetic acid was added and samples were incubated for 15 min. An equal amount of 100 µL of pre-chilled 50 µL buffer (80% acetonitrile, 0.1% formic acid in H₂O) was added to 100 µL of each sample. Samples were mixed gently on a plate mixer for 30 s, centrifuged at 14 000 g for 5 min and supernatant was aspirated. 100 µL of ice-cold 70% acetonitrile 0.1% formic acid was added to each sample, gently mixed and centrifuged at 14 000 g for 5 min. The supernatant was aspirated and pellets were resuspended in 50 µL of ACN (80% acetonitrile, 0.1% formic acid in H₂O) and centrifuged at 14 000 g for 5 min. The supernatant was aspirated and pellets were resuspended in 50 µL of lysis buffer and incubated at 4 °C for 10 min. 1.5 mL of urea buffer A (80% acetonitrile, 0.1% formic acid in H₂O) and spun for 1 min at 14 000 g. The wash step was repeated twice. Filters were washed with 100 µL ABC (0.05 M ammonium bicarbonate) and spun for 1 min at 14 000 g. The wash step with ABC was performed three times. Filters were then transferred to a new collecting tube, and 40 µL of ABC with trypsin (1:100 enzyme to protein ratio) was loaded onto the filter. This was mixed at 600 rpm in a thermomixer for 1 min. Filters were sealed with parafilm to prevent evaporation and incubated at 37 °C o/n. After trypsin digestion, filters were centrifuged for 10 min at 14 000 g. 50 µL of 0.5 M NaCl was added and filters were centrifuged for 10 min at 14 000 g. 4 µL of trifluoroacetic acid (TFA) was added to acidify samples. Samples were then subjected to stage tip preparation. Stage tips were generated by stacking 200 µL pipet tips with three layers of C18. Tips were washed with 100 µL MeOH and spun for 2 min at 2500 g, washed with 100 µL Buffer B (80% acetonitrile, 0.1% formic acid in H₂O) and spun for 1 min at 2500 g. Samples were loaded onto stage tips and centrifuged for 4 min at 1500 g. Tips were washed with 100 µL Buffer A and spun for 2 min at 2500 g, which was repeated once. Samples were then eluted in 40 µL Buffer B, speedvacced to 5 µL, and filled up to 12 µL with buffer A.

Mass Spectrometry Analysis: 5 µL digested peptides was injected into an Easy-nLC1000 (Thermo) connected online to an LTQ-Orbitrap-Fusion mass spectrometer (Thermo) by developing a gradient from 7 to 30% Buffer B for 214 min before washes at 60% then 95% Buffer B, for 240 min of total data collection time. The flow rate was 250 nL min⁻¹. Full MS scans were collected from 400 to 1500 m/z with an Orbitrap resolution of 120 000 and an AGC target of 3e5. MS/MS spectra were recorded in the lon trap using higher-energy collision dissociation fragmentation. The ion trap scan rate was set at Rapid. An AGC target of 2e4 was used with HCD collision energy at 30% and an intensity threshold of 1e4. Scans were recorded in data-dependent top-speed mode of a 3-s cycle with dynamic exclusion until all recoveries are 50% or 15 min 10 min of data collection is reached. The mass tolerance for precursor ions was set to 20 ppm and the mass tolerance for fragment ions to 0.5 Da. The match between runs feature was enabled and LFQ and IBAQ values were calculated for each protein. The output Proteingroups file containing all detected proteins was loaded into Perseus.[53] Proteins were first filtered against a reverse and contaminant database. Next, the conditions were grouped in Perseus and any protein that was not detected in all replicates of a single condition was discarded. Missing values were imputed from the random distribution with default parameters (width = 0.3, Down shift = 1.8). The proteins in the resulting list were annotated as chromatin-associated or not chromatin associated (Table S2, Supporting Information). This was done by comparing to a list of factors that were experimentally and in vivo shown to be associated with chromatin (enriched in non-pluripotent human cells; we converted the names to mouse names and we manually included known mouse pluripotency factors). In addition, we called all zinc finger proteins chromatin-associated as these are known to possess nucleic acid binding domains. To specifically
identify transcription factors, our detected proteins were matched with two published lists of murine transcription factors. Correlation between replicates was assessed using spearman correlation. Proteins that were significantly different between the conditions were assessed using ANOVA statistics with Benjamini–Hochberg correction for multiple testing. Proteins were called significant with FDR < 0.05. To calculate p-values in pairwise comparisons in Figure 3B, we used Welch's t-test. A list of all detected proteins and whether these are significantly different between the conditions can be found in Table S2, Supporting Information.

For comparison of ChEP with whole cell proteomes, we used whole cell proteomes that were generated previously in our lab. Downstream analysis was done with R, Python3, and Jupyter Notebook. GO analysis was performed using DAVID.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011782.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
G.v.M. and R.A.W. contributed equally to this work. The authors thank Cristina Furlan for technical advice regarding the ChEP procedure and discussions concerning mass spectrometry. Research in the group of HM is supported by a grant from the Netherlands Organization for Scientific Research (NWO-VIDI 864.12.007).

Conflict of Interest
The authors declare no conflict of interest.

Keywords
chromatin, differentiation, mouse embryonic stem cells, proteomics, pluripotency

Received: April 28, 2019  
Revised: June 7, 2019  
Published online: July 3, 2019

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