Citrullination is the post-translational conversion of an arginine residue within a protein to the non-coded amino acid citrulline[1]. This modification leads to the loss of a positive charge and reduction in h

a clear reduction in reprogramming (Fig. 2a,b, Extended Data Fig. 5b-e and Extended Data time-lapse video). Consistent with this, levels of *Tcl1* and *Nanog* were not elevated upon reprogramming to the same extent as in control cells (Fig. 2b and Extended Data Fig. 5f). Cl-amidine treatment led to a dramatic reduction of reprogramming efficiency and H3Cit (Fig. 2c and Extended Data Fig. 5g-i), suggesting that the catalytic activity of PADI4 is important for the induction of pluripotency.

Padi4 expression and H3Cit are detected in the early embryo[20,21] and *Padi4-null* mice are born in lower numbers than would be expected by Mendelian inheritance[22], suggesting that PADI4 loss affects embryonic development. To assess the role of PADI4 in early development, we cultured mouse embryos in Cl-amidine-containing medium from the 2-cell stage and th yiNghout res-mpolnt aion oevelopment,(FseeMenthodsand Extended Data Fig. 5]TJ T* [(i6a CTis,led to a deducte Hpercnt,ageof pluripotenct anogvi15 (el epiblastoells d Ean)]TJ T* [(imnceatse Hp

and significantly increased upon PADI4 over-expression), it is refractory to modification when Arg54 is mutated (Fig. 3f). Similar results were obtained *in vitro* citrullination assays (Extended Data Fig. 11a). H1R54 lies within the globular domain of H1 (Extended Data Fig. 11b), which is highly conserved among the linker histone family and is necessary for interaction with nucleosomal DNA[27,28]. To test whether H1R54 is necessary for binding of H1 to nucleosomes, we mutated and assessed it in nucleosome-binding assays. Figure 3g shows that an R54A mutant, which mimics the charge change that accompanies citrullination, is impaired for nucleosome binding. An R54K mutant, which retains the positive charge, is impaired to a lesser extend (Fig. 3g) suggesting that H1R54 is important for electrostatic interactions between H1.2 and the nucleosome.

The above results open up the possibility that PADI4 may affect chromatin compaction in pluripotent cells. To test this hypothesis, we first assessed whether citrullination by ectopic PADI4 can lead to decondensation of differentiated cell chromatin. Recombinant PADI4 was added to permeabilized and stabilized differentiated C2C12 mouse myoblast nuclei (Fig. 4a). This protocol ensures stabilization of the nuclear component while allowing the free diffusion of non-chromatin bound nuclear proteins into the extra-nuclear fraction, and their collection by washing. Incubation with active PADI4 (Extended Data Fig. 12a,b), leads to the eviction of H1 from the chromatin and its diffusion out of the permeabilized nucleus (Fig. 4b). The evicted H1 is citrullinated on R54, as determined by mass spectrometry (Extended Data Figure 12c,d). Consistent with this, PADI4-treated cells showed evidence of decondensed chromatin, as determined by nuclear swelling, diffuse DAPI staining and increased sensitivity to micrococcal nuclease (Fig. 4c,d and Extended Data Figure 12e). Similar results were observed when *PADI4* was over-expressed in C2C12 cells (Fig. 4e) or NS cells (data not shown). To monitor if PADI4 can affect H1 binding on pluripotent cell chromatin, we performed ChIP-qPCR analyses of H1.2 on the regulatory regions of *Oct4* and *Nanog* and found that it is stabilized upon *Padi4* knockdown (Fig. 4f). The ability of PADI4 to disrupt the binding of H1 to nucleosomal DNA provides a novel mechanistic example of how citrullination regulates protein function and chromatin condensation.

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Extended Data

Extended Data Figure 1:

(a) Transcript levels for *Padi1, Padi2 and Padi3* in ES, NS and iPS cells, as assessed by qRT-PCR. *Padi6* was undetectable in all three cell types. Expression normalized to endogenous levels of *Ubiquitin (UbC)* Error bars represent the standard error of the mean of three biological replicates.

(b) Transcript levels of *Padi1, Padi2 and Padi3* in ES cells upon switch to 2i containing medium for one passage, as assessed by qRT-PCR. *Padi6* was undetectable in both conditions. Expression normalized to *UbC*. Error bars represent the standard error of the mean of three biological replicates.

(c) Immunoblot analysis of H3Cit levels in ES, NS and iPS cells. Total H3 is presented as loading control.

(d) Immunoblot analysis of total citrullination levels in ES, NS and iPS cells, using an antibody against Modified Citrulline (ModCit) which recognizes peptidylcitrulline irrespective of amino acid sequence. Total H3 is presented as loading control.
(e) ZHBTc4.1 and 2TS22C ES cell lines were treated with 1 g/ml doxycycline for 48 hours, resulting in depletion of Oct4 or Sox2 (data not shown). *Padi4* mRNA was significantly reduced upon Oct4, but not Sox2 knockdown, as assessed by qPCR. Error bars represent standard error of the mean of four biological replicates.
(f) ChIP-qPCR for Oct4, Sox2, Klf4, RNA polymerase II (PolII), H3K4me3 and H2A on the promoter of *Padi4* in mES and NS cells. For each cell condition, the signal is presented as fold enrichment over Input and after subtracting background signal from the beads. Error bars represent the standard deviation of three technical qPCR replicates.
Asterisks denote difference with ES cells (a) or media (b), and 0h time point (e); - not significant, * P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001 by ANOVA (a) or t-test (b,e).

Extended Data Figure 2:
(a) Validation of selected targets from the PADI4 over-expression microarray dataset by qRT-PCR. Expression of *Pou5f1*, *Sox2*, *Klf4* and *c-Myc* is not affected by PADI4 over-expression. Expression normalised to *UbC*. Error bars presented as standard error of the mean of three biological replicates.
(b) Transcript levels of mouse *Padi4* and human *PADI4* in mES cells after transient knock-down with *Padi4* or control (Ctrl) shRNA, and over-expression of human *PADI4* or control

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vector (pPB CTRL), as assessed by qRT-PCR. Expression normalized to *Hprt*. Error bars represent the standard error of the mean of three biological replicates.
(c) Transcript levels of mouse *Padi4, Tcl1* and *Nanog* in mES cell clones stably expressing *Padi4* or control (Ctrl) shRNA, as assessed by qRT-PCR. Expression normalized to *Hprt*. Error bars represent the standard error of the mean of three biological replicates. Asterisks denote difference with Ctrl (a,b,c) and between samples (b); - not significant, * P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001 by ANOVA (b) or t-test (a,c).

Extended Data Figure 3:
(a) Representative ChIP-qPCR for H2A on regulatory regions of *Tcl1* and *Nanog* in mES, NS and iPS cells (corresponding to Fig. 1h). For each cell condition, the signal is presented as fold enrichment over Input and after subtracting background signal from the beads. Error

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Extended Data Figure 4:

(a) Heat map of the top 70 genes that showed differential expression after PADI4 inhibition in ES cells by with 200 M Cl-amidine for 48h, as determined by microarray analysis. Displayed values are normalized log intensities, minus the mean expression of the gene across the two samples. Hierarchical clustering based on correlation.

(b) Validation of selected targets from the above microarray dataset by qRT-PCR. Expression normalised to *Gapdh*. Error bars presented as standard error of the mean of three

biological replicates. Asterisks denote difference with Ctrl; - not significant, * P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001 by t-test.
(c) Gene Ontology for Biological Process (GOBP) analysis for the mutated gene categories within the microarray dataset of Cl-amidine treatment in mES cells. *P*-value is corrected for multiple testing using Benjamini and Hochberg FDR.

Extended Data Figure 5:
(a) Scheme of reprogramming of neural stem cells to pluripotent state. NSO4G cells were retrovirally transduced with Oct4, Klf4 and c-Myc. After 6 days, partially reprogrammed pre-iPS cells arose. For shRNA experiments, pre-iPS cells were stably transfected with Ctrl or PADI4 shRNA and then full reprogramming was performed in the presence of 2iLIF media for 8 days. For PADI4 enzymatic inhibition, pre-iPS cells were immediately changed to 2iLIF media in the presence of the inhibitor Cl-amidine for 8 days.
(b) Quantification of flow cytometry analysis for the assessment of Oct4-GFP reporter expression in a reprogramming assay using pre-iPS cells stably expressing *Padi4* shRNA #4 and Ctrl shRNA. Error bars represent standard error of the mean of triplicate samples within a representative from four reprogramming experiments.
(c) Quantification of Oct4-GFP positive colonies in the reprogramming assay where pre-iPS cells were *Padi4* shRNA #4 versus control (see Fig. 2a), after time-lapse image acquisition with Biostation CT. Error bars represent standard error of the mean of triplicate samples within a representative reprogramming experiment. Time-lapse video in supplementary data online.
(d) Immunoblot analysis of H3Cit in pre-iPS cells treated with 2i, after *Padi4* knock-down

(e) Quantification of flow cytometry analysis for the assessment of Oct4-GFP reporter expression in a reprogramming assay using pre-iPS cells stably expressing *Padi4* shRNA #3 and Ctrl shRNA. Error bars represent standard error of the mean of triplicate samples.
(f) qRT-PCR analysis for the expression of *Tcl1*, *Nanog* and *Padi4* mRNAs at the end of the above reprogramming assay (e). Error bars represent standard error of the mean of triplicate samples.
(g) Quantification of flow cytometry analysis for the assessment of Oct4-GFP reporter expression in a reprogramming assay were treated with 200 M Cl-amidine. Error bars represent standard error of the mean of triplicate samples within a representative from three reprogramming experiment.
(h) Quantification of Oct4-GFP positive colonies in the reprogramming assay where pre-iPS cells were treated with 200 M Cl-amidine (see *Fig.* 2c) after time-lapse image acquisition with Biostation CT. Error bars represent standard error of the mean of triplicate samples within a representative reprogramming experiment.
(i) Immunoblot analysis for the presence of H3Cit at the end of the reprogramming assay (g). Total histone H3 presented as loading control.
Asterisks denote difference with Control; - not significant, * P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001 by t-test

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Extended Data Figure 6:

(a) Embryos at 2-cell stage were treated with 200 M Cl-amidine and snapshots were tak at E3.0, E3.5 and E4.0. 200 M Cl-amidine embryos arrested at 8-cell stage, while Control embryos continued development to form blastocysts. Phase contrast images are shown.

(b) Embryos at 2-cell stage were treated with 10 M Cl-amidine for 12 hours, fixed and stained for H3Cit at the 4-cell stage. Phase contrast, H3Cit (white) and HOECHST 33342 (blue) images are shown. Bar represents 20 m.

(c) Embryos at E3.5 were treated with 10 M Cl-amidine for 24 hours, fixed and stained for H3Cit at E4.5. H3Cit (green) and HOECHST 33342 (blue) images are shown. Bar represents 20 m.

(d) Table with quantifications of lineage commitment in E4.5 blastocysts treated with 10 M Cl-amidine from the 2-cell stage. Asterisks denote difference with Control, unpaired t-test, * = p<0.05. n=3 (50 embryos).
(e) Embryos were cultured in medium supplemented with 10 M Cl-amidine from 2-cell stage and through preimplantation development. E4.5 blastocysts were fixed and stained for SOX17 (primitive endoderm marker, red), Cdx2 (trophectoderm marker, green) and HOECHST 33342 (blue). Bar represents 20 m.
(f) Time-lapse analysis of distribution of inner and outer cells at the 8 to 16-cell transition, upon culturing of embryos with medium containing 10 M Cl-amidine from 2-cell stage. Error bars represent standard error of the mean. Statistical significance was determined by unpaired t-test or Mann Whitney test upon non-normal distribution. Asterisks denote difference with Control; * P 0.05.

Extended Data Figure 7:
(a) Embryos at 2-cell stage were treated with 100 M TDFA for 12 hours and fixed and stained for H3Cit at 4-cell stage. H3Cit and HOECHST 33342 images are shown.
(b) Table representing the percentage of cells committed to each embryonic lineage in E4.5 blastocysts upon treatment of embryos at 2-cell stage with 100 M TDFA. Bars represent mean percentage (SEM). Asterisks denote difference with Control, Mann-Whitney test, * = p<0.05. n=3 (60 embryos).
(c) Embryos at 2-cell stage were treated with 100 M TDFA and fixed at embryonic day E4.5. Phase contrast, Nanog (green), Sox17 (purple), Cdx2 (red) and HOECHST 33342 (blue) images are shown.

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Extended Data Figure 8:

(a) Embryos at 2-cell stage were treated with 10nM TSA for 12 hours, and fixed and stained for H3K9ac at 4-cell stage. H3K9ac and HOECHST 33342 images are shown.

(b) Table representing the percentage of cells committed to each embryonic lineage in E4.5 blastocysts upon treatment of embryos at 2-cell stage with 10nM TSA. Bars represent mean percentage (SEM). Asterisks denote difference with Control, unpaired t-test, * = $p<0.05$. n=2 (32 embryos).

(c) Embryos at 2-cell stage were treated with 10nM TSA and fixed at embryonic day E4.5. Phase contrast, Nanog (green), Sox17 (purple), Cdx2 (red) and HOECHST 33342 (blue) images are shown.

Extended Data Figure 9:

(d)

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Extended Data Figure 10:

(a) MS spectrum of Histone H1.5 in a SILAC proteomic screen for identification of PADI4 substrates. Linker histone H1.5 is deiminated by PADI4, as identified by a highly increased SILAC ratio of the heavy labeled identified peptide (marked by a red dot).

(b) Fragmentation spectra of the doubly charged LysC peptide ERGGVSLPALK surrounding Arginine 54 of H1.5. The y and b series indicate fragments at amide bonds of the peptide, unambiguously verifying the citrullinated peptide.

Extended Data Figure 11:

(a) Mutation of R54 renders histone H1.2 refractory to deimination. Immunoblot analysis of recombinant histone H1.2 using an antibody that detects all deimination events (ModCit). Wild type and R54-mutant H1.2 were treated with recombinant PADI4, in the presence of activating calcium. Only wild-type H1.2 can be deiminated, indicating that R54 is the only substrate of PADI4 in H1.2. No-calcium reactions presented as negative controls. Total H1.2 presented as loading control.

(b) Schematic representation of the position of R54 within the globular domain linker histone H1.2.

Extended Data Figure 12:

(a) Immunoblot analysis of the Pellet fraction of C2C12 permeabilised cells treated with recombinant PADI4. Presence of H3Cit species indicates PADI4 activity. Total H3 is presented as a control for equal use of starting material in the two experimental conditions.

(b) Immunofluorescence analysis of C2C12 nuclei after treatment with recombinant hPADI4. Presence of H3Cit species indicates PADI4 activity. DNA is visualised by staining with DAPI.

(c) Fragmentation spectra of the citrullination site R54 on the evicted Histone H1.2 peptide ERSGVSLAALK (corresponding to Fig. 4b). The evicted Histone H1 is citrulinated at R54.

(d) Theoretical and measured b- and y-ion fragment masses for the citrullinated H1.2 peptide (peptide sequence ERSGVSLAALK) detected after treatment of C2C12 cells with recombinant hPADI4 (corresponding to Fig. 4b).

(e) Micrococcal nuclease digestion of C2C12 nuclei after treatment with recombinant PADI4, as described in Fig. 4a. M= size marker.

Supplemental Information

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: PADI4 expression and activity are features of pluripotent cells

(a,b) qRT-PCR for *Padi4* and *Nanog* expression in ES, NS and iPS cells (a), and in ES cells upon culture in 2i/LIF for one passage (b). *Pou5f1, Olig2* and *Pax6* are presented as controls. Expression normalized to *ubiquitin (UbC)*. Error bars: standard error of the mean of three biological replicates.

(c) qRT-PCR for *Padi4* and *Nanog* expression and H3Cit immunoblot during the course of reprogramming (see also Extended Data Fig. 5a). Loading control: total histone H3. Representative of four experiments.

(d) Heat map of the genes regulated upon *hPADI4* over-expression in mES cells, as determined by microarray analysis. Displayed values are normalized log intensities, minus the mean expression of the gene across the four samples. Hierarchical clustering based on correlation.

(e) Gene Ontology for Biological Process (GOBP) analysis of the microarray dataset. *P*-value is corrected for multiple testing using Benjamini and Hochberg False Discovery Rate (FDR).

(f,g) qRT-PCR for *Tcl1* and *Nanog* expression in mES cells after transient knock-down with *Padi4* or control (Ctrl) shRNAs, and over-expression of human *PADI4* or control vector (pPB CTRL) (f), and after treatment with 200 M Cl-amidine (g). Expression normalized to *UbC*. Error bars: standard error of the mean of three biological replicates.

(h) ChIP-qPCR for H3Cit on regulatory regions of *Tcl1* and *Nanog* in mES, NS and iPS cells. Error bars: standard deviation of three technical qPCR replicates. Representative of three experiments.

Asterisks denote difference with ES cells (a) or media (b), Control (f, g) and between samples (f); - not significant, * P 0.05, ** P 0.01, *** P 0.001, **** P 0.0001, by ANOVA (a,f) or t-test (b,g).

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Figure 2: Citrullination and PADI4 regulate pluripotency during reprogramming and early embryo development

(c) Flow cytometry analysis and phase contrast/fluorescence images for the assessment of Oct4-GFP reporter expression after reprogramming assay of pre-iPS cells treated with 200 M Cl-amidine. Representative of three independent experiments.
(d) E4.5 blastocysts from 2-cell stage embryos treated with 10 M Cl-amidine. SOX17 (primitive endoderm marker, red), Cdx2 (trophectoderm marker, green), Nanog (epiblast marker, white) and HOECHST 33342 (blue).
(e) Distribution of inner cell mass versus trophectoderm cells in E3.5 blastocyst treated as above.

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Figure 3: PADI4 citrullinates Arg54 on linker histone H1 and affects its binding to nucleosomal DNA

(a) Experimental strategy for screening for PADI4 citrullination substrates in the chromatin fraction of ES cells.

(b) Scatter plot representing the overall fold change for all identified citrullination sites. Red diamonds: PADI4-regulated citrullinations.

(c) Table representing the 40 most highly regulated PADI4 substrates, their individual citrullination sites and the $\log_2$ SILAC ratio. Complete dataset in Supplementary Table 4.

(d) Quantification of citrullination site R54 on H1.2 through differential regulation of the triply charged peptide ERSGVSLAALKK.

(e) Fragmentation spectra of the triply charged light and heavy SILAC labeled LysC peptide ERSGVSLAALKK surrounding Arginine 54 of H1.2. The y and b series indicate fragments at amide bonds of the peptide.

(f) Citrullination immunoblot of wild-type and R54A mutant GFP-tagged H1.2 expressed and pulled-down from ES cells expressing *PADI4* or control vector (Mock). Control for the efficiency of the pull-down: GFP.

(g) Nucleosome pull-down assay using wild-type and R54-mutant H1.2. WB: Western Blot.