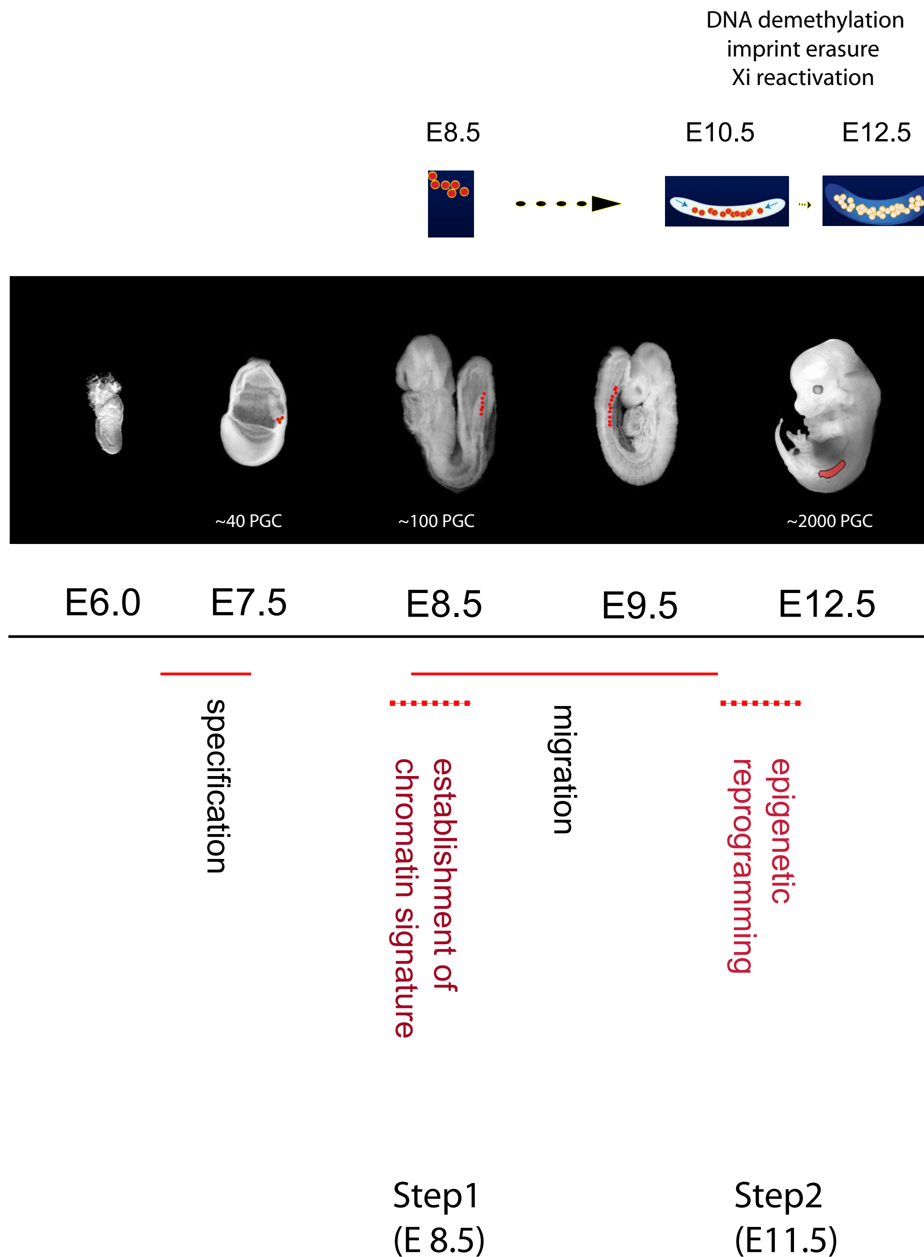
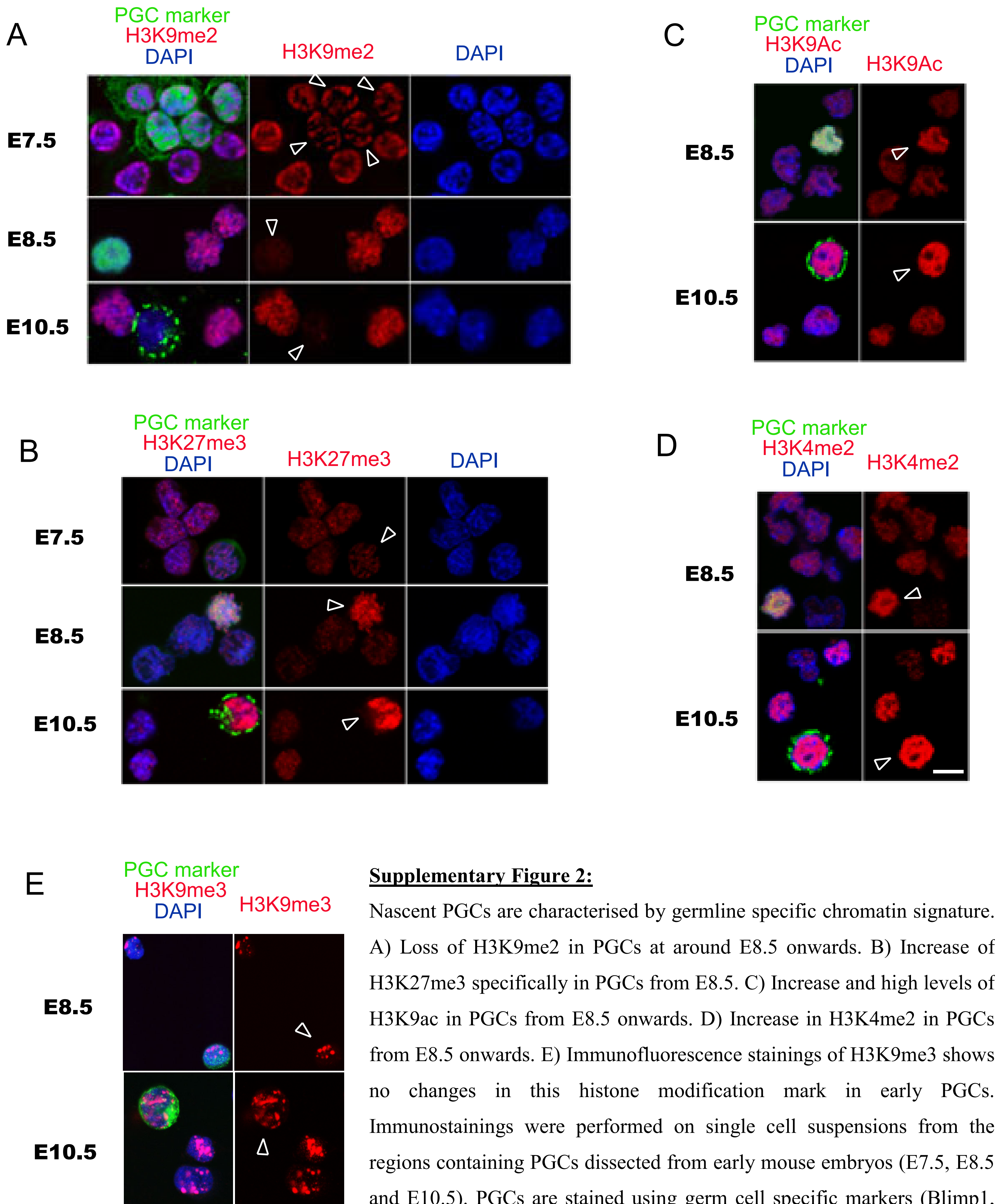
doi: 10.1038/nature06714

Suppl.Fig.1



Supplementary Figure 1:

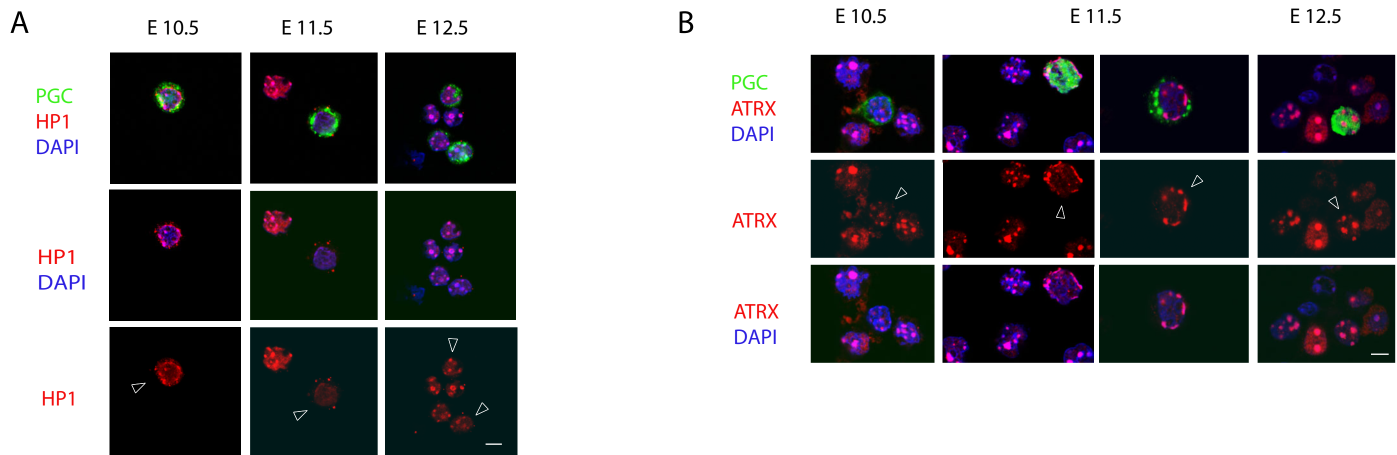
Overview of PGC development in the mouse. The nascent PGCs are first identified on E7.5 as a group of about 40 Stella expressing cells. On E8.5, (Step1 of the reprogramming process) when there are about 100 PGCs per embryo, they start to migrate along the developing hindgut and reach the developing gonads at about E10.5. Soon after the entry into the gonads the PGCs undergo epigenetic reprogramming (as a second step of the reprogramming process), which includes genome-wide DNA demethylation, erasure of genomic imprints and re-activation of the inactive X chromosome (Xi) in female embryos.



<u>Supplementary Figure 2:</u>

Nascent PGCs are characterised by germline specific chromatin signature. A) Loss of H3K9me2 in PGCs at around E8.5 onwards. B) Increase of H3K27me3 specifically in PGCs from E8.5. C) Increase and high levels of H3K9ac in PGCs from E8.5 onwards. D) Increase in H3K4me2 in PGCs from E8.5 onwards. E) Immunofluorescence stainings of H3K9me3 shows no changes in this histone modification mark in early PGCs. Immunostainings were performed on single cell suspensions from the regions containing PGCs dissected from early mouse embryos (E7.5, E8.5 and E10.5). PGCs are stained using germ cell specific markers (Blimp1, Oct4 or SSEA1) in green and are depicted by arrows. Scale bar: 10µm.

Suppl. Fig. 3



Supplementary Figure 3:

Disappearance and relocalisation of heterochromatin associated proteins in PGCs at E11.5. A) Presence and localisation HP1α. The data shows loss of HP1 staining in PGCs at E11.5. B) Detection of ATRX. Note the relocalisation of ATRX to the nuclear periphery in PGCs at E11.5. Stainings were performed on single cell suspensions from genital ridges between E10.5-E13.5. The shown epigenetic changes are detectable in 70-90% of PGCs depending on the developmental stage of the embryo. Germ cells were stained in green using the germ cell specific markers SSEA1 and Oct4, respectively, and are depicted by arrows. Scale bar: 10µm.

Suppl.Fig.4

A

E 10.5 E 11.5 E 12.5

Oct4
H3S28P
DAPI

H3S28P

B

E 10.5 E 11.5 E 12.5

Oct4
H3S10P
DAPI

H3S10P

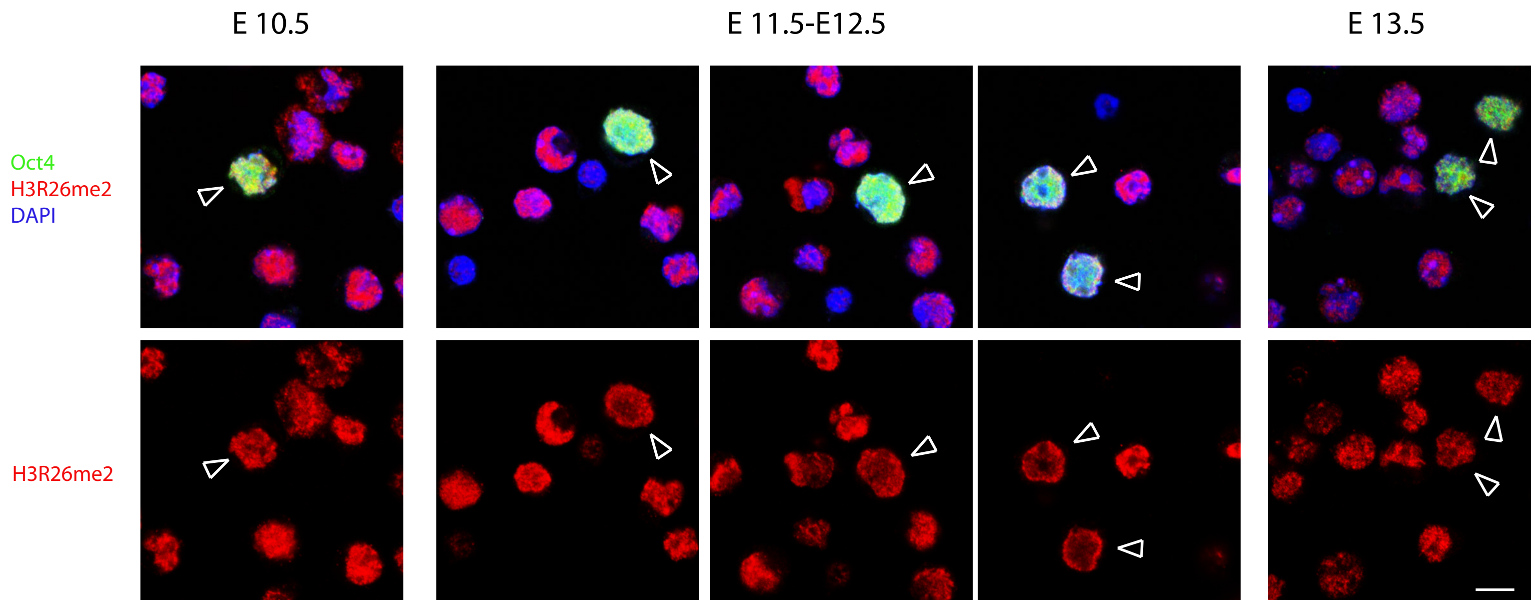
H3 - N terminal tail:

A-R-T-K-Q-T-A-R-K-S-T-G-G-K-A-P-R-K-Q-L-A-T-K-A-A-R-K-S-A-P~~~~~~~~
9 27

Supplementary Figure 4

Phosphorylation of H3S28 and H3S10 is not detectable in gonadal PGCs. (A). Immunofluorescence analysis of phosphorylation of H3S28 phosphorylation in single cell suspension from genital ridges. High levels of phosphorylation are detectable in mitotic cells. PGCs identified by Oct4 antibody (in green). Scale bar: 1μm. (B). Immunofluorescence detection of phosphorylation of H3S10 phosphorylation in single cell suspension from genital ridges. High levels of H3S10 phosphorylation are detectable in mitotic cells. PGCs identified by Oct4 antibody (in green). Scale bar: 10μm.

Suppl.Fig.5



H3 - N terminal tail:

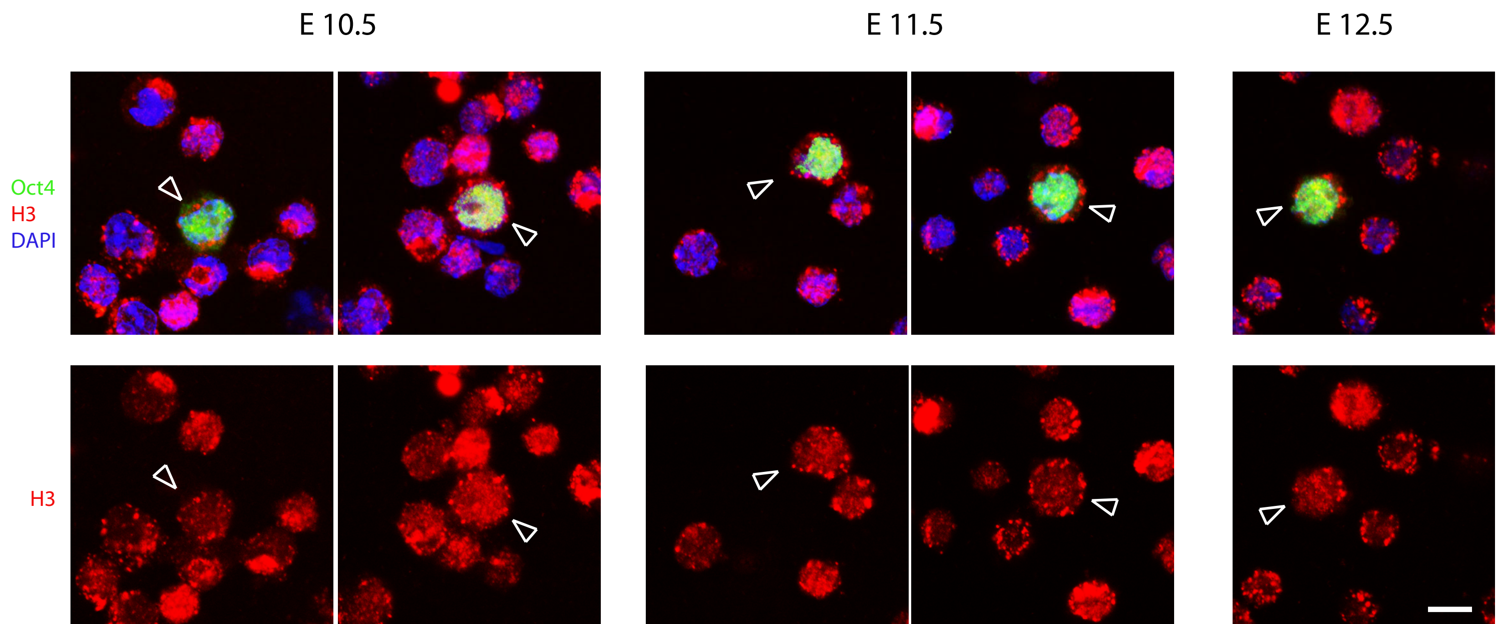
A-R-T-K-Q-T-A-R-K-S-T-G-G-K-A-P-R-K-Q-L-A-T-K-A-A-R-K-S-A-P~~~~~~~~

9 27

Supplementary Figure 5

Disappearance of H327me3 signal in PGCs is not caused by changes in H3R26 methylation. Immunofluorescence detection of H3R26me2 in single cell suspension from genital ridges. Note the changes in the localisation of signal in PGCs during reprogramming. However, PGCs do not show accumulation of this modification in the nucleus at this time and therefore cannot account for the disappearance of H3K27me3 signal. PGCs identified by Oct4 antibody (in green). Scale bar: 10µm.

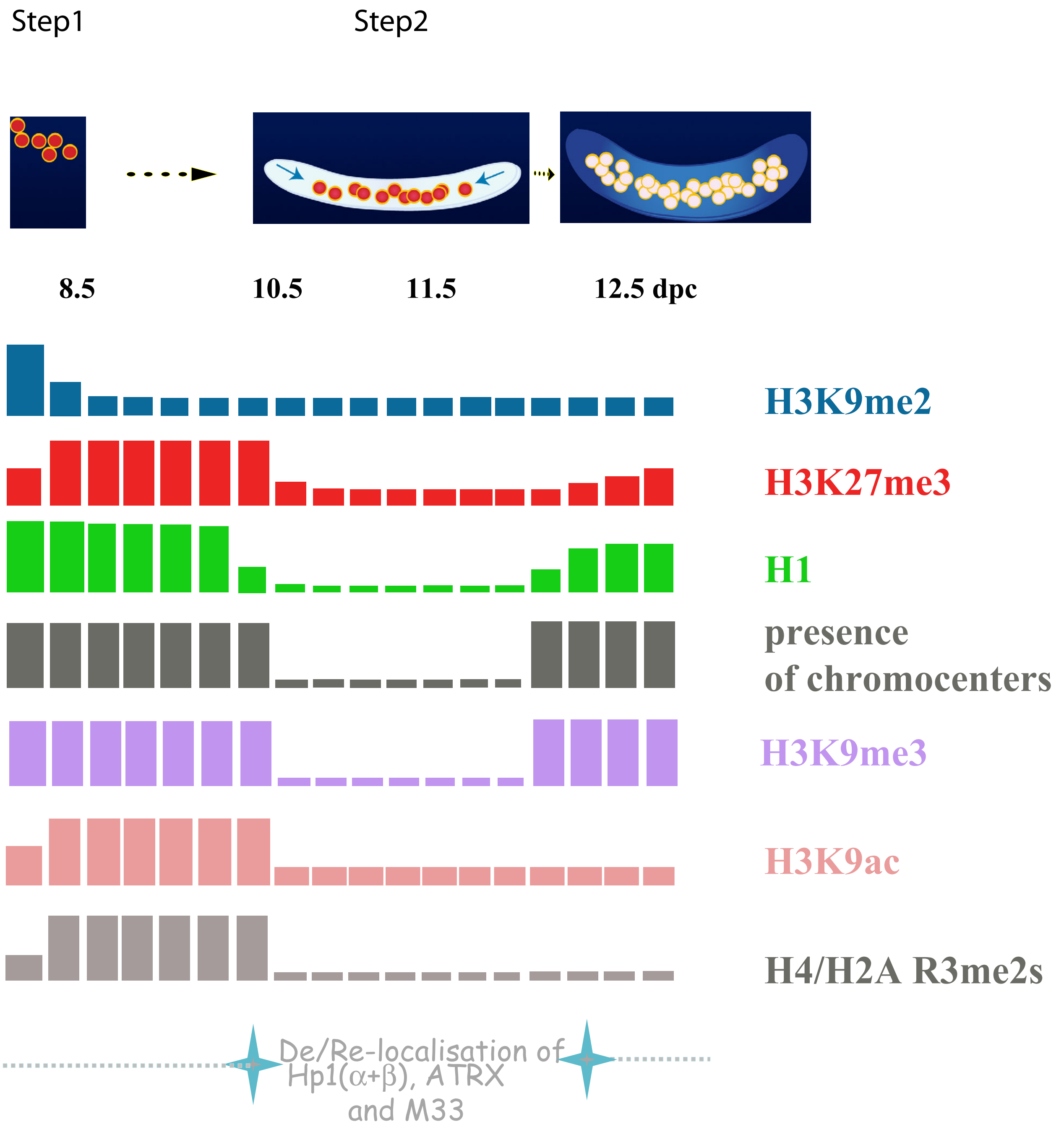
Suppl.Fig.6



Supplementary Figure 6

Histone H3 is detectable in PGCs throughout the reprogramming process. Immunostaining of histone H3 in single cell suspension prepared from genital ridges. The PGCs are staind by Oct4 antibody (green). Please note continuous presence of the signal for H3 in PGCs. Scale bar: 10µm.

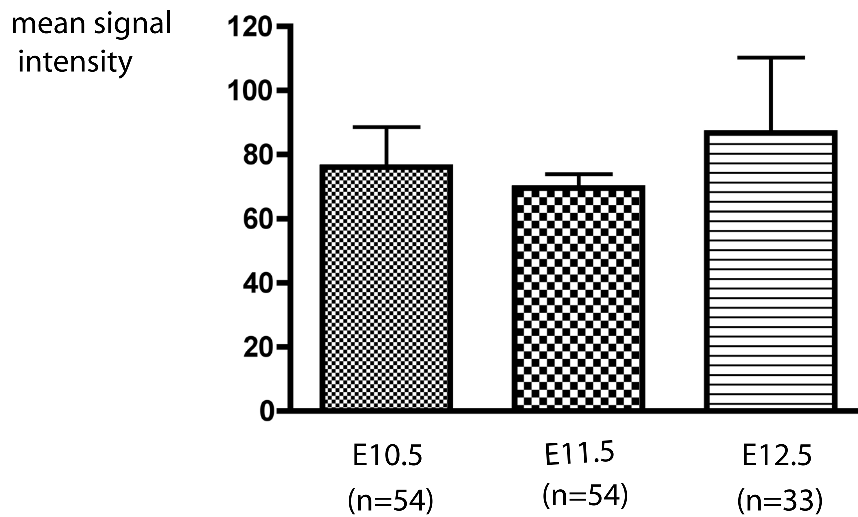
Suppl. Fig. 7



Supplementary Figure 7

Scheme summarising chromatin changes occurring in PGCs during the reprogramming process. The chromatin changes in PGCs occur in 2 steps. First step is characterized by loss of H3K9me2 and gain of H3K27me3, H3K9ac and H4/H2A R3me2s at E8.5. The second step occurs at E11.5 is characterized by changes in nuclear architecture (loss of chromocenters) and by the loss of numerous histone modifications (for details see the main text).

Suppl.Fig.8



Supplementary Figure 8

Quantification of H2A.Z signal in somatic cells of the genital ridges. Note that there is no significant change in the signal between E10.5-E12.5 in somatic cells. The y-axis values are arbitrary and represent the mean signal intensity.

Suppl. Fig. 9

A

cyclin B1 | cyclin D1 | merge (+DAPI)

E11.5

GFP

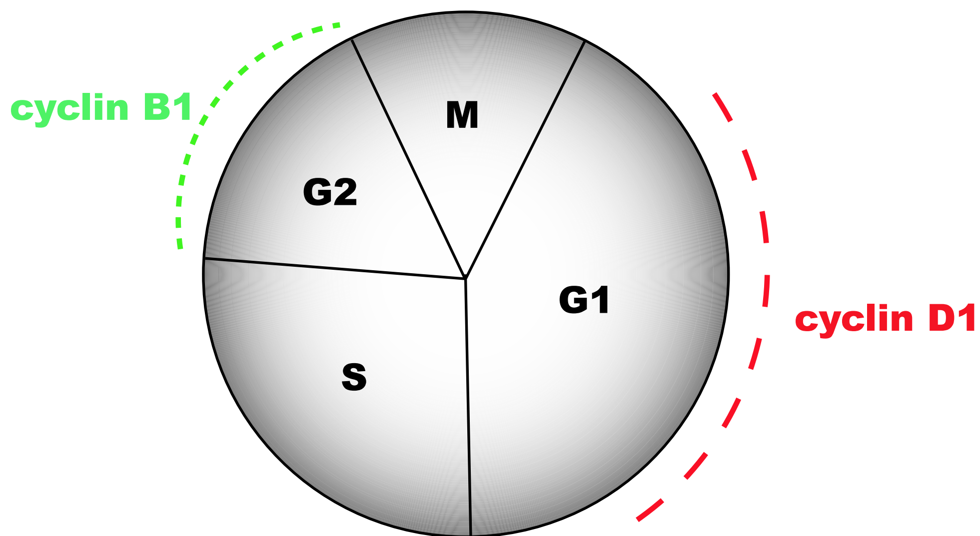
B

A

100%

93%

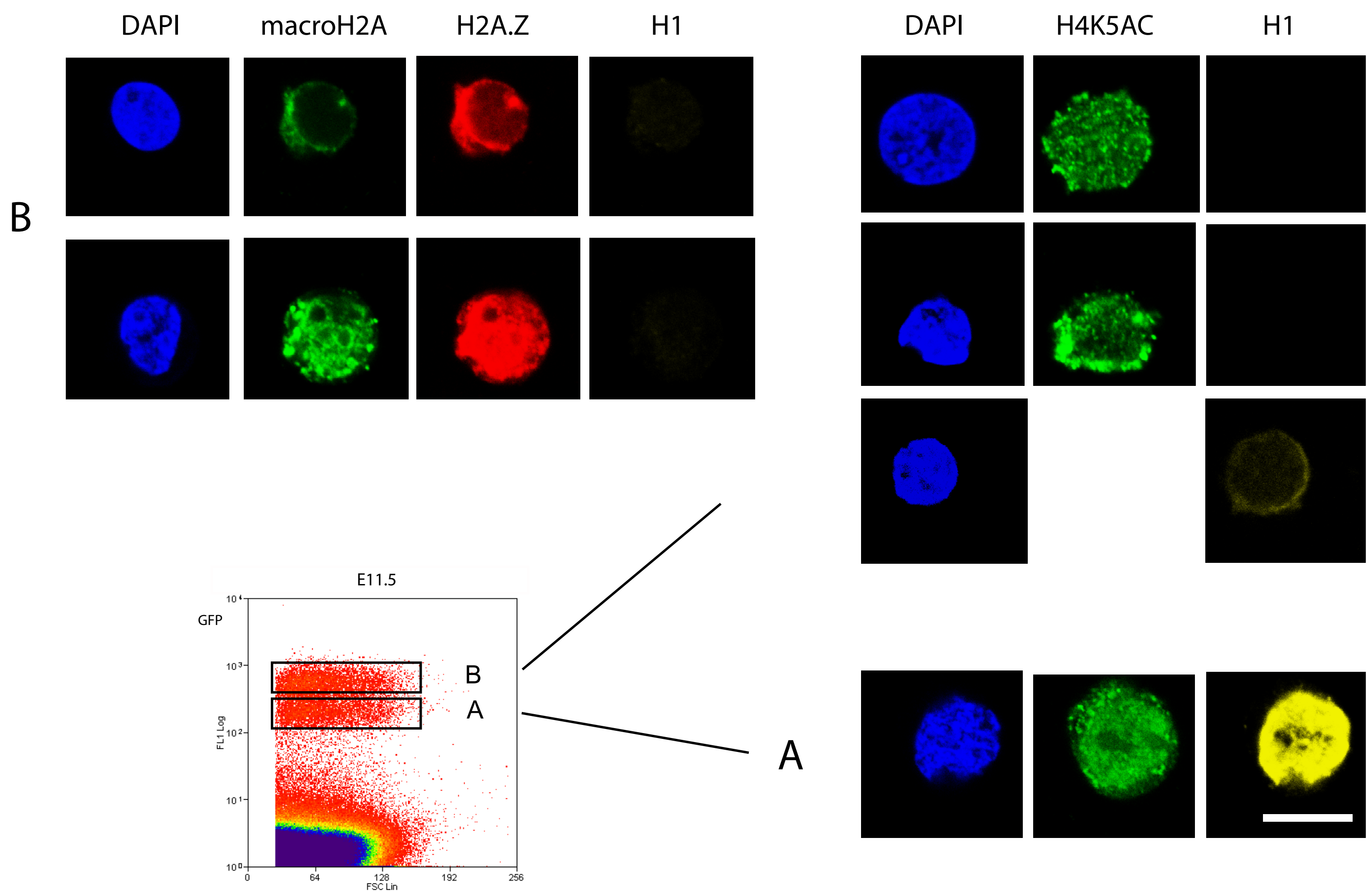
B



<u>Supplementary Figure 9:</u>

PGCs undergoing DNA demethylation and chromatin remodelling are in G2 phase of the cell cycle. A) Immunofluorescence staining of FACS sorted PGCs of populations A and B show absence of cyclin D1 and the nuclear localisation of cyclin B1. Scale bar: 10μm. B) Schematic representation of the cyclin B1 and D1 expression during the cell cycle.

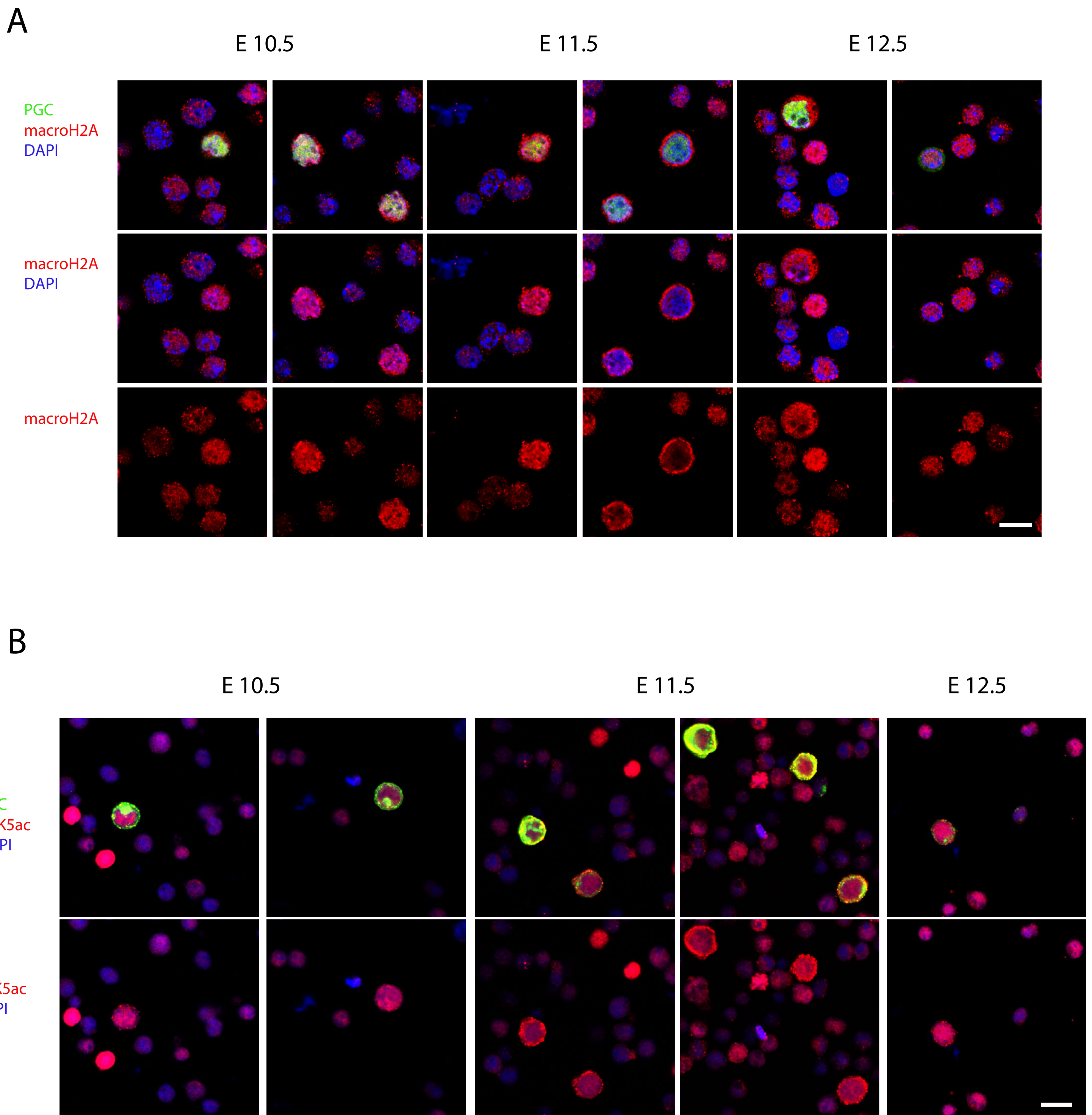
Suppl. Fig. 10



Supplementary Figure 10:

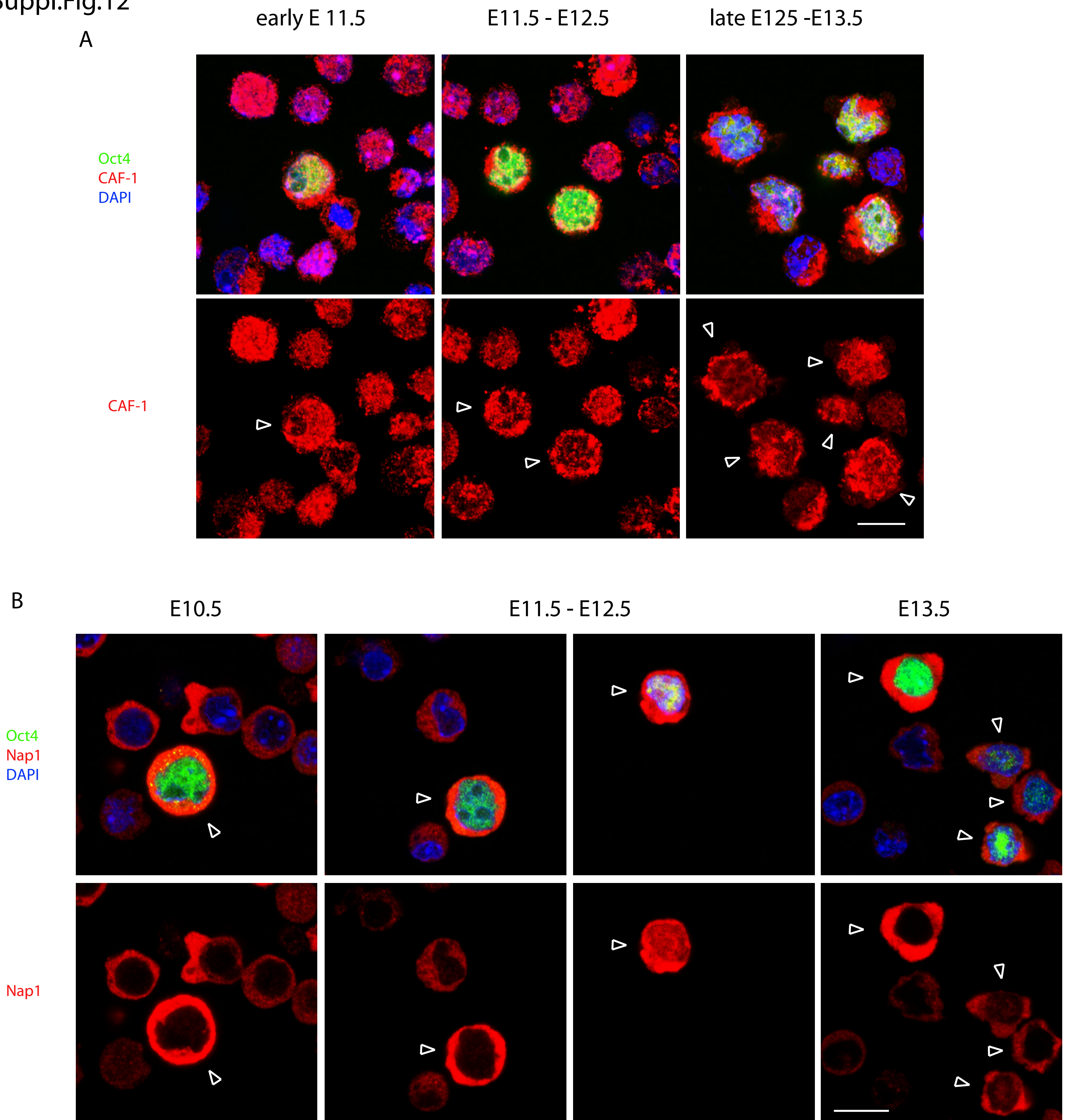
Cytoplasmic localisation of histones during the reprogramming process. Prior to complete disappearance of H1 and H2A.Z stainings in FACS sorted PGCs of population B it is possible to detect signals for these histones in the cytoplasm. Additionally, we were able to detect transient cytoplasmic localisation of macroH2A and H4K5ac. (See also Suppl. Fig. 11) Scale bar: 10µm.

Suppl. Fig.11



Supplementary Figure 11:

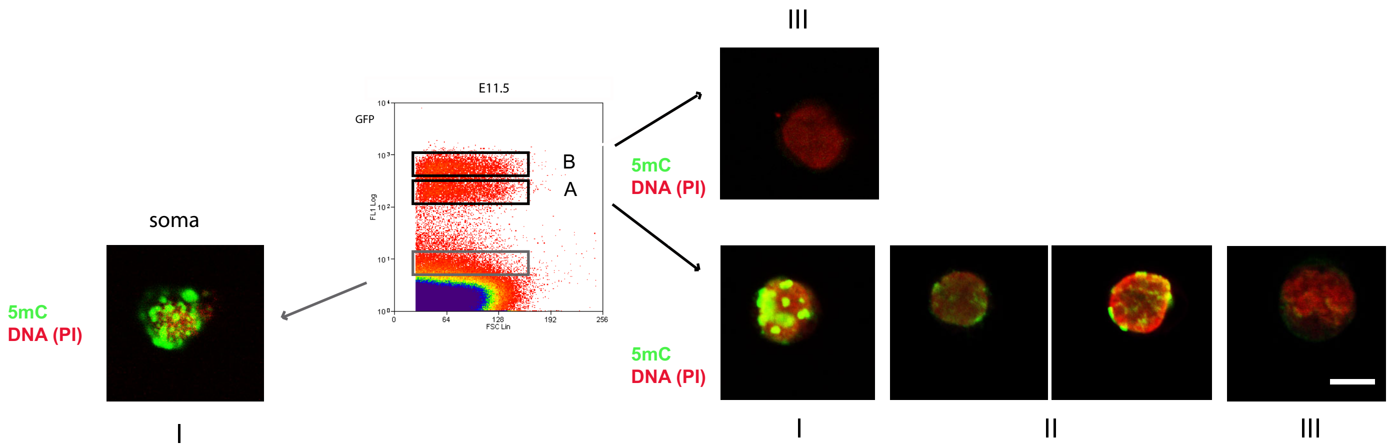
Cytoplasmic localisation of macroH2A (A) and H4K5ac (B) in PGCs undergoing reprogramming. Stainings were performed on single cell suspensions from genital ridges between E10.5-E13.5. Germ cells were stained in green using the germ cell specific markers SSEA1 and Oct4, respectively. Scale bar: 10µm.

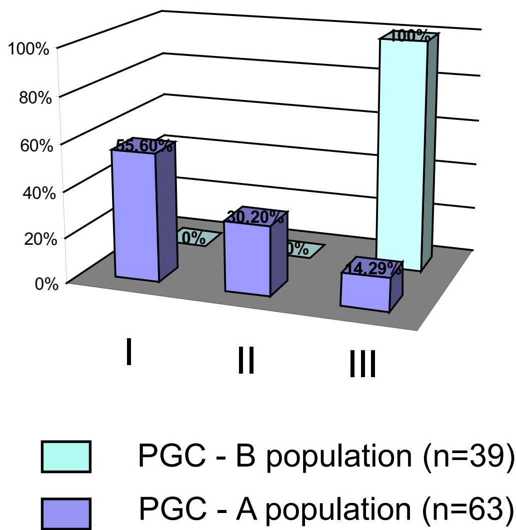


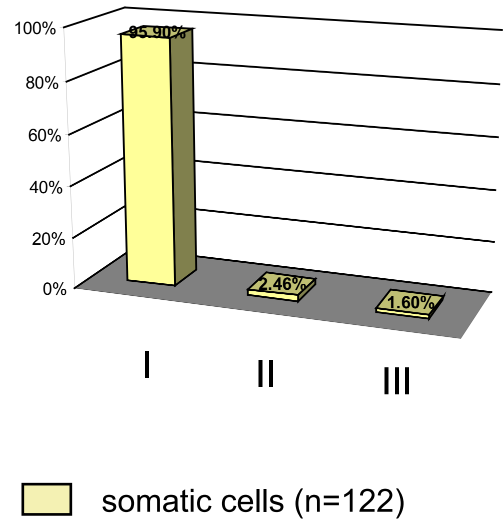
Supplementary Figure 12:

Presence and localisation of some of the histone chaperones in PGCs from E10.5-E13.5. A) Changes of CAF-1 (p150) localisation during PGC development. CAF-1 is nuclear in gonadal PGCs up to early E11.5. The data shows transient relocalisation of CAF-1 to the cytoplasm in some PGCs between E11.5 and E12.5. Note that the population of PGCs in embryonic gonad is not developmentally synchronous. B) Gonadal PGCs are characterised by high levels of histone chaperone NAP1. In PGCs, NAP1 is cytoplasmic until mid/ late E11.5, which is followed by transient re-localisation to the nucleus between E11.5 and E12.5. Germ cells were identified as Oct4 positive in green and are depicted by arrows. Scale bar: 10µm.

Suppl. Fig .13







Supplementary Figure 13:

Kinetics of DNA demethylation process in E11.5 PGCs as documented by 5mC staining. A) 5mC immunofluorescence staining of FACS sorted PGCs and surrounding somatic cells. Note the presence of cells with somatic type (type I) as well as intermediate level (type II) of 5mC staining alongside with cells devoid of 5mC staining (type III) in population A. PGCs of population B are demethylated and are devoid of 5mC staining (type III). The left panel shows 5mC staining in somatic cells of the genital ridges. Scale bar: 10µm B) Quantification of cells showing different 5mC staining patterns (type I-III) in A and B populations of PGCs and C) surrounding somatic cells.

Supplementary Methods

Embryo collection and PGC preparation

PGCs were isolated from outbread MF1 mice. Noon of the day of the vaginal plug was designated as E0.5. For the FACS sorting the dissected urogenital ridges from embryos carrying *Oct4-GFP* transgene were trypsinized and treated with hyaluronidase prior to the FACS sorting using the MoFlo (Cytomation Bioinstruments). The purity of PGCs was examined independently using Oct4 staining and was always in excess of 98%.

Immunofluorescence staining

The urogenital ridges of the relevant developmental stages were trypsinised and the single cells suspension settled on the poly-L-lysine (Sigma-Aldrich) treated slides. The cells were briefly washed with 1xPBS and fixed in 4%PFA in PBS for 15minutes at room temperature. The cells were permeabilised for 30 min using PBS, 1%BSA, 0.1% Triton TX-100. The antibody staining was carried out in the same buffer at 4°C overnight. The slides were subsequently washed 3x in PBS, 1%BSA, 0.1% Triton TX-100 (5min each wash) and incubated with Alexa dyes conjugated secondary antibodies (Molecular Probes) for 1 hr at room temperature in dark, washed 1x 5 min in PBS, 1%BSA, 0.1% Triton TX-100 and 2x 5 min in PBS. The slides were then mounted in Vectashield with DAPI (Vector laboratories) and imaged using BioRad Radiance 2100 confocal microscope. The quantification of nuclear size and staining intensity was performed on cryosection (see below) using Velocity software.

For cryosections the dissected urogenital ridges (E12.5 and E13.5) or trunks of embryos (E10.5 and E11.5) were fixed in 4%PFA (or in 2%PFA for H2A.Z stainings) for 4 hrs at 4°C, washed 3x 10min in PBS and incubated in 15% sucrose (in PBS) overnight at 4°C. The material was then mounted in OCT matrix (Cell Path) and stored in -80°C until cryo-sectioning. The cryosections were post-fixed with 4%PFA in PBS for 5 min, washed 3x 5min in PBS, permeabilised and processed further as above.

5methylcytosine staining

The FACS sorted cells of populations A, B and the somatic cells of the genital ridges were allowed to attach to the poly-L-lysine (Sigma-Aldrich) treated slides for 15 min at room temperature. Following the fixation with 4%PFA in PBS for 10min, the cells were washed 3x 5min in PBS and permeabilised in PBS, 1%BSA, 0.5% TX-100 for 30 min. The slides were then washed with PBS and treated with 4N HCl for 20 or 30min (PGCs or somatic cells, respectively) at 37°C. Following extensive PBS washes, the slides were blocked in PBS, 1%BSA, 0.1%TX-100 for 30min and hybridised in the same buffer with 5mC antibody at 4 ^{o}C overnight. The slides were subsequently washed 3x in PBS, 1%BSA, 0.1% Triton TX-100 (5min each wash) and incubated with Alexa dyes conjugated secondary antibodies (Molecular Probes) for 1 hr at room temperature in dark, washed 1x 5 min in PBS, 1%BSA, 0.1% Triton TX-100 and 2x 5 min in PBS, followed by RNaseA treatment (1mg/ml – 30min) and propidium iodide (PI) staining (0.25mg/ml – 10min). The slides were then mounted in Vectashield (Vector laboratories) and imaged as above.

Antibodies

H2AZ (kind gift of D. Tremethick) 1:200 (the specificity of the antibody was tested on the AUT gels – data not shown); H1 (Abcam) 1:200; ATRX (Santa Cruz Biotechnologies) 1:50; H3K9me2 (Upstate) 1:400; H3K9me3 and H3K9me27 (kind gift of T. Jenuwein) 1:500; H3K9ac (Abcam) 1:300; Nap1l1 (Abcam) 1:200; H3K4me2 and H3K4me3 (Abcam) 1:500; HIRA (kind gift of P. Adams) combination of monoclonal antibodies; CAF-1 (anti p150 – kind gift of G. Almouzni) 1:100, 5mC antibody (kind gift of A.Nivelau) 1:50)

Bisulphite sequencing

The FACS sorted PGCs were embedded in SeaPlaque LMP agarose and processed as described previously .Nested PCR approach was used for the PCR amplification of *peg3* and *lit1* DMR regions as in [1]. For *Peg3* :Primers: F1: ttt tta gat ttt gtt tgg ggg ttt tta ata, R1: aat ccc tat cac cta aat aac atc cct aca, F2: ttg ata ata gta gtt tga ttg gta ggg tgt, R2: atc tac aac ctt atc aat tac cct taa aaa . The PCR conditions: 1st PCR (primers F1,R1) : 95°C 5min, 95 ^{o}C 1min, 58 ^{o}C 75sec, 72 ^{o}C 60sec (35cycles) 72 ^{o}C 10min, 4 ^{o}C . 2nd PCR (primers F2, R2) : 95°C 5min, 95 ^{o}C 1min, 58 ^{o}C 75sec, 72 ^{o}C 60sec (35cycles) 72 ^{o}C 10min, 4 ^{o}C. For *lit1* : Primers: F1: tat tat ttt ggt gtt ggt tat atc ggg

tta, R1 : att ttt ctt caa cac cct tct ttt ccc t, F2 : ggg tta taa agt tta ggg gtt ttt aga tt, R2 : aaa ctt ttc tat tca act taa ttc cca ac. The PCR conditions: 1st PCR (primers F1,R1) : 95°C 5min, 95 °C 1min, 57 °C 75sec, 72 °C 60sec (35cycles) 72 °C 10min, 4 °C . 2nd PCR (primers F2, R2) : 95°C 5min, 95 °C 1min, 57 °C 75sec, 72 °C 60sec (35cycles) 72 °C 10min, 4 °C.

The PCR products were gel purified, cloned into a T/A cloning vector (pGEM Easy System I, Promega) and transformed into Top10 ultracompetent *E.coli* (Invitrogen). The inserts were verified using colony PCR and sequenced using external sequencing service.