# Haploid mouse germ cell precursors from embryonic stem cells reveal *Xist* activation from a single X chromosome

Eishi Aizawa[1], Corinne Kaufmann[1], Sarah Sting[1], Remo Freimann[1], Anton Wutz[1,*]

[1]Institute of Molecular Health Sciences, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland

*Correspondence: awutz@ethz.ch

## Abstract

In mammals, one of the two X chromosomes of diploid female cells is inactivated for gene dosage compensation between the sexes. *Xist* initiates X chromosome inactivation (XCI) in female cells. Although *Xist* is not expressed in male cells with a single X chromosome, it remains unclear if a single X chromosome in a haploid cell would lead to *Xist* activation. Haploid embryonic stem cells (haESCs) have been derived from several mammalian species [1], and maintain pluripotency in culture. They also show a predisposition for diploidization especially during differentiation. Establishing somatic haploid cells has been reported by inhibiting potential factors for diploidization [2, 3]. Considering high diploidization in somatic lineages, we wondered if specification of germline progenitor cells could be less restrictive to a haploid genome. Here, we report that mouse primordial germ cell-like cells (PGCLCs) preferentially maintain a haploid genome. Using this differentiation system, we find that *Xist* is initially induced in differentiating haESCs but subsides later after germ cell specification. The observation that XCI is initiated in haploid cells

demonstrates *Xist* activation without the requirement of a second X chromosome inactivation center (*Xic*).

To perform germ cell differentiation we followed a previously established protocol [4]. A mixed population of haploid and diploid ESCs was differentiated to epiblast-like cells (EpiLCs) for 2 days, followed by aggregation into embryoid bodies (EBs) (Figure 1A, S1A). In this culture PGCLCs arose between day 6 to 8, and were identified by co-expression of SSEA1 and integrinβ3 (Figure 1B, S1B). To investigate the ploidy of the cells we stained the DNA with Hoechst and analyzed the cells by flow cytometry. G0/G1/S-phase haploid (1n) cells accounted for 9.3% of day 7 EB cells, which is approximately half the fraction at the beginning of differentiation (16.8%; Figure S1C). We further noted that the 1n haploid population contained substantial numbers of cells that expressed both SSEA1 and integrinβ3 (Figure 1B). PGCLCs accounted for 32.3% of G0/G1/S-phase haploid cells. However, less than 10% of cells with higher DNA content expressed both PGC markers (Figure 1D, S1D, S1E). Conversely, haploid cells were enriched in the PGCLC population compared to SSEA1- and integrinβ3-negative cells that likely represent somatic fates (Figure S1F). We confirmed a set of 20 acrocentric chromosomes in haploid PGCLCs as expected for a haploid mouse genome (Figure 1C). Furthermore, the PGC markers, *Blimp1*, *Prdm14*, *Tfap2c* and *Stella*, were upregulated, and *Dnmt3b* was downregulated in sorted PGCLCs relative to EpiLCs and ESCs (Figure S2A). Our results show that haploid PGCLCs can be obtained *in vitro* whereby a haploid genome is preferentially maintained in PGCLCs over somatic lineages.

We next used this differentiation system to address the question if *Xist* is activated

47 from a single X chromosome upon differentiation of haESCs. We sorted G0/G1/S-phase
48 haploid (1n) and S/G2/M-phase diploid (4n) cells at different timepoints during germ cell
49 differentiation of a mixed haploid-diploid ESC culture and performed RNA FISH for
50 detecting *Xist* RNA. At the start of differentiation, we observed one and two foci in haploid
51 and diploid ESCs, respectively (Figure S2B), which correspond to nascent *Xist* and *Tsix*
52 expression before initiation of XCI. In EpiLCs, *Xist* clusters were observed indicating the
53 activation of *Xist*. A single *Xist* cluster was observed in 42% and 59% of haploid and diploid
54 EpiLCs, respectively (Figure 1E, F). Surprisingly, 17.9% of diploid EpiLCs had two *Xist*
55 clusters. This observation is unexpected and might be explained by a potential deregulation
56 of XCI when the cell was in a haploid state. We confirmed a strong upregulation of *Xist* by
57 quantitative RT-PCR analysis in both haploid and diploid EpiLCs (Figure S2A). Taken
58 together, our data demonstrate that *Xist* can be activated from a single X chromosome at
59 the onset of haESC differentiation. With further differentiation we observed that the
60 proportion of cells exhibiting *Xist* clusters decreased in haploid and diploid d7 PGCLCs.
61 How this decrease is achieved is unclear, but repression of *Xist* in germ cell differentiation
62 or death of *Xist* expressing cells are possible mechanisms.

63 The persistence of haploid cells during germ cell differentiation enabled us to perform
64 an analysis of *Xist* activation in the context of a haploid genome. Previous studies reported
65 *Xist* activation in genomes of different ploidies [5]. Tetraploid cells transiently activate *Xist*
66 on a variable number of X chromosomes and a stochastic regulation has been proposed.
67 In contrast, observations on pairing of X chromosomes at their *Xic* loci has been reported
68 and implicated in the activation of *Xist* [6, 7]. Furthermore, elements that are required for
69 *Xic* pairing have been identified and transgenically shown to induce XCI [8]. In haploid cells,

*Xic* pairing cannot occur as only a single X chromosome is present similar to male cells. In contrast to diploid male cells, we find that a single X chromosome is sufficient for *Xist* activation in haploid cells. Therefore, *Xist* activation can occur independent of *Xic* pairing. Our finding does not rule out that *Xic* pairing could have a role in ensuring that one X chromosome remains active in female cells. We further note that our interpretation is consistent with a recent study that has used tethering of the *Xic* regions to the nuclear lamina to prevent *Xic* pairing [9]. Although it is hard to rule out in this study that *Xic* pairing had occurred at some point, the report that XCI could be initiated when *Xic* movement was constraint to the lamina is consistent with our observation.

We also find that PGCLCs are obtained from haESCs with higher frequency than somatic lineages. This observation might reflect a similar epigenetic state of PGCs and ESCs. In mice, PGCs initiate reactivation of the inactive X chromosome and genome-wide DNA demethylation during migration to the gonads. A lack of dosage compensation and low DNA methylation level are also associated with naive ESC and the early epiblast [10]. Therefore, it is conceivable that ESCs and PGCs are less restricted for ploidy and relative X-linked gene expression. Haploid PGCLCs will be useful for future studies of the genetics of germ cell development.

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## Author Contributions

E.A. and A.W. conceptualized experiments. E.A., C.K., S.S. and R.F. collected the data. E.A. and C.K. analyzed the data. E.A. and A.W. wrote the manuscript.

## Declaration of Interests

The authors declare no competing interests.

## Figure Legends

**Figure 1. Preferential maintenance of haploidy in PGCLCs and *Xist* activation during germ cell differentiation.** (A) A scheme of germ cell differentiation of mixed haploid-diploid ESCs. (B) Flow cytometry analysis of DNA content of diploid ESCs, mixed haploid-diploid ESCs, and d7 EBs derived from mixed haploid-diploid ESCs. Cell cycle phases (top), and PGCLCs in d7 EBs, positive for SSEA1 and integrinβ3, are indicated (green). (C) Representative chromosome spread of a haploid d7 PGCLC. (D) The ratio of PGCLCs in cell populations of different DNA content in d7 EBs derived from mixed haploid-diploid ESCs or diploid ESCs. PGCLCs accounted for 32.3% on average out of G0/G1/S haploid cells in d7 EBs derived from mixed haploid-diploid ESCs. Data represents the mean and the standard error of the mean. n = 18 (d7 EBs derived from mixed haploid-diploid ESCs) and n = 12 (d7 EBs derived from diploid ESCs). **** $P < 0.0001$. (E) Representative images of *Xist* expression in G0/G1/S-phase haploid EpiLCs and PGCLCs detected by RNA FISH using a Cy3-labeled *Xist* probe (orange). Single *Xist* clusters (arrow) and foci (arrowhead) were observed in G0/G1/S-phase haploid EpiLCs. Single foci but no cluster were observed in G0/G1/S-phase haploid PGCLCs. (F) Proportion of *Xist* RNA FISH signals during germ

115 cell differentiation of mixed haploid-diploid ESCs. The number of cells possessing no,
116 single or double *Xist* RNA clusters were counted in G0/G1/S-phase haploid and S/G2/M-
117 diploid cells. Total numbers of counted cells are 138 (H, ESC), 171 (D, ESC), 100 (H,
118 EpiLC), 173 (D, EpiLC), 69 (H, PGCLC) and 121 (D, PGCLC). D, S/G2/M-phase diploid;
119 dip, diploid; H, G0/G1/S-phase haploid; hap, haploid
120

121 # Supplemental Information

122 Supplemental Information includes experimental procedures, two figures and one table.
123

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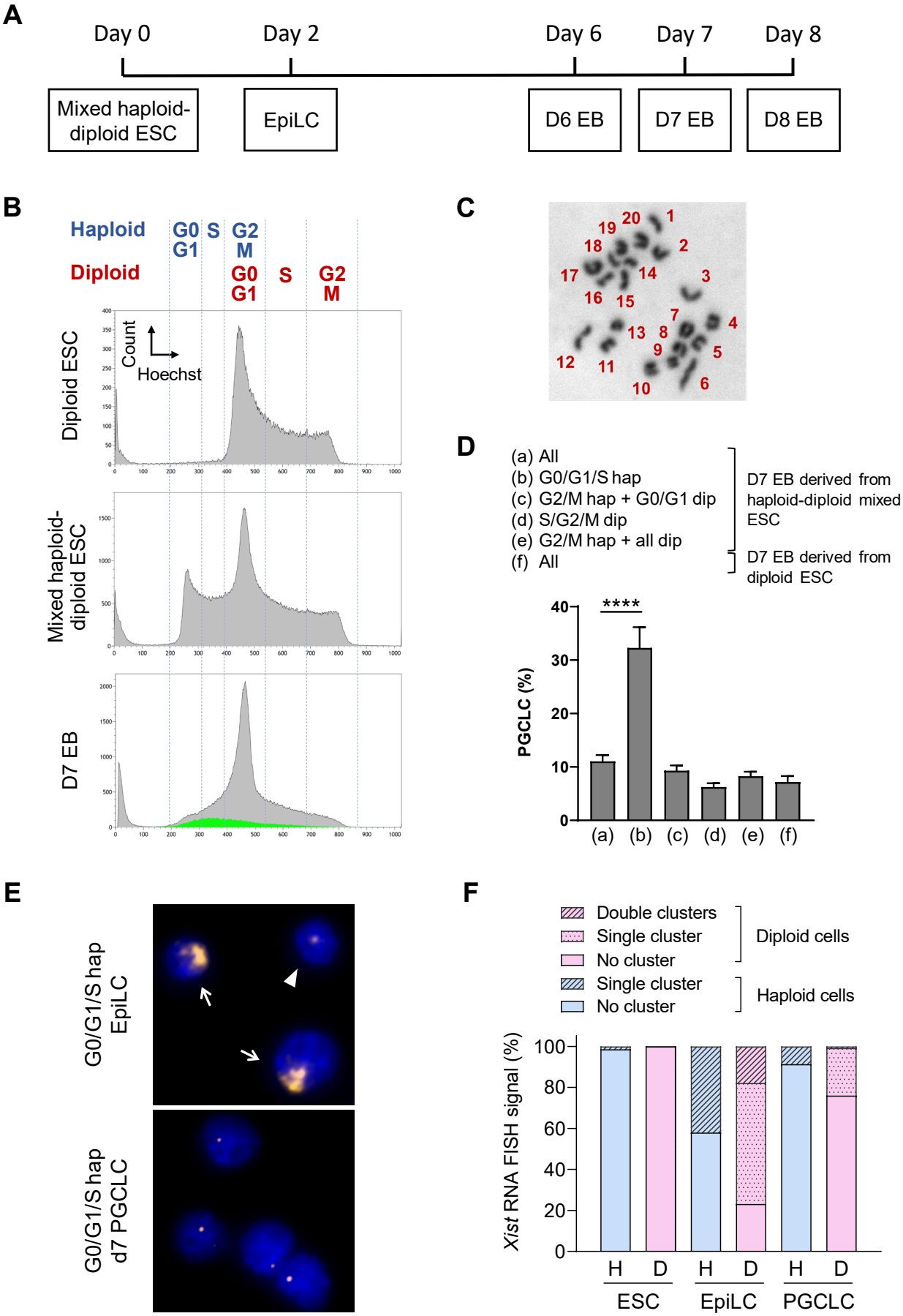
A
Day 0
Day 2
Day 6
Day 7
Day 8
Mixed haploid-diploid ESC
EpiLC
D6 EB
D7 EB
D8 EB
B
Haploid
G0 G1
S
G2 M
Diploid
G0 G1
S
G2 M
Diploid ESC
Count
Hoechst
Mixed haploid-diploid ESC
D7 EB
C
D
(a) All
(b) G0/G1/S hap
(c) G2/M hap + G0/G1 dip
(d) S/G2/M dip
(e) G2/M hap + all dip
(f) All
D7 EB derived from haploid-diploid mixed ESC
D7 EB derived from diploid ESC
PGCLC (%)
****
(a) (b) (c) (d) (e) (f)
E
G0/G1/S hap EpiLC
G0/G1/S hap d7 PGCLC
F
Double clusters
Single cluster
No cluster
Diploid cells
Single cluster
No cluster
Haploid cells
Xist RNA FISH signal (%)
H D H D H D
ESC EpiLC PGCLC