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Transcriptional and epigenetic control of germline competence and specification

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In mammals, germline specification is induced during early embryogenesis when competent cells respond to extrinsic signals and form primordial germ cells (PGCs), the precursors of the gametes. The fusion of the two types of gametes, the egg and the sperm, gives rise to a new organism and closes the germline cycle. With the entry of the germline, the PGCs are separated from the soma and thus ensure the self-perpetuation of the species. Using the mouse as a model of mammalian embryogenesis, in this review we will focus on the transcriptional and epigenetic changes that regulate the initial steps of germline development, namely germline competence and PGC specification.

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Introduction

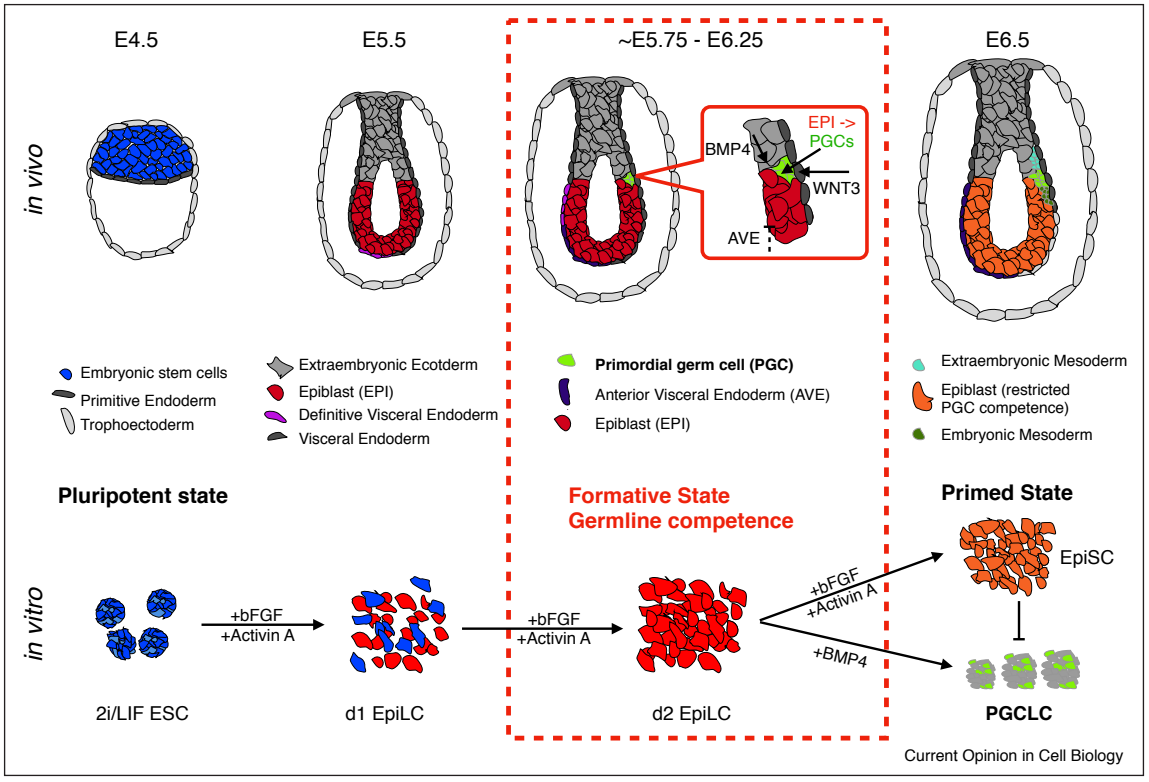
To safeguard the transmission of genetic information to the next generation, primordial germ cells (PGCs) are separated from the soma during early development. In some animals, like *Drosophila*, *Xenopus or Zebrafish*, PGCs are specified by maternally inherited determinants soon after fertilization (preformation). In mammals, PGCs are specified later during embryogenesis (after implantation), as cells get exposed to inductive signals (epigenesis) [1]. Germline competent cells are responsive to those inductive signals and therefore the key to understand the origin of mammalian germ cells. Germline competence and PGC specification cannot be studied *in vivo* in humans due to ethical limitations and, consequently, these processes are best understood in the context of mouse embryogenesis. Nevertheless, recent advances in the understanding of human germline development have revealed many similarities but also important differences between mice and humans (reviewed in Refs. [2–4]).

Following implantation and the exit from naïve pluripotency (E4.5–E5.5), PGC specification is initiated in a few epiblast cells at the proximo-posterior end of the mouse embryo (E6.25) [5] in response to inductive signals emanating from extraembryonic tissues: WNT3 from the visceral endoderm and BMP4 from the extraembryonic ectoderm (Figure 1) [6,7]. By E7.25, a population of approximately 40 PGCs are formed at the base of the allantois [5], which will then undergo migration into the genital ridges, expansion and epigenomic reprogramming. The PGC phase of germline development concludes by E13.5, as germ cells enter meiosis and gametogenesis [8]. Here, we will focus on the acquisition of germline competence by the post-implantation mouse epiblast cells (E5.5–E6.25) and their subsequent specification into PGCs (E6.25–E7.25). The molecular events occurring during this period have been historically difficult to investigate *in vivo*, due to the limited tractability of mouse peri-implantation development. These limitations can be circumvented by using an *in vitro* differentiation system that faithfully recapitulates PGC specification (Figure 1) [9] or by applying single-cell and low-cell genomic technologies *in vivo* [10•,11••,12,13]. In the following sections we will summarize how recent *in vitro* and *in vivo* studies are rapidly improving our view of the major transcriptional and epigenetic events that regulate early PGC development.

Transcriptional changes during early PGC development

During the transition from naïve to formative pluripotency, epiblast cells undergo major transcriptional changes. Particularly, the naïve expression program is shut down (e.g. *Prdm14*, *Esrrb*) [12,14], while early post-implantation markers are induced (e.g. *Fgf5*, *Otx2*) [15]. In response to inductive signals, a few epiblast cells located at the proximo-posterior end of the embryo revert these changes and initiate PGC specification by reactivating the vast majority of the naïve pluripotency programme. In contrast, most epiblast cells acquire a somatic fate by transitioning to a primed pluripotent state, in which early somatic lineage specifiers become activated and germline competence is lost. Remarkably, general pluripotency factors (i.e. *Pou5f1*, *Sox2*) remain expressed

Figure 1



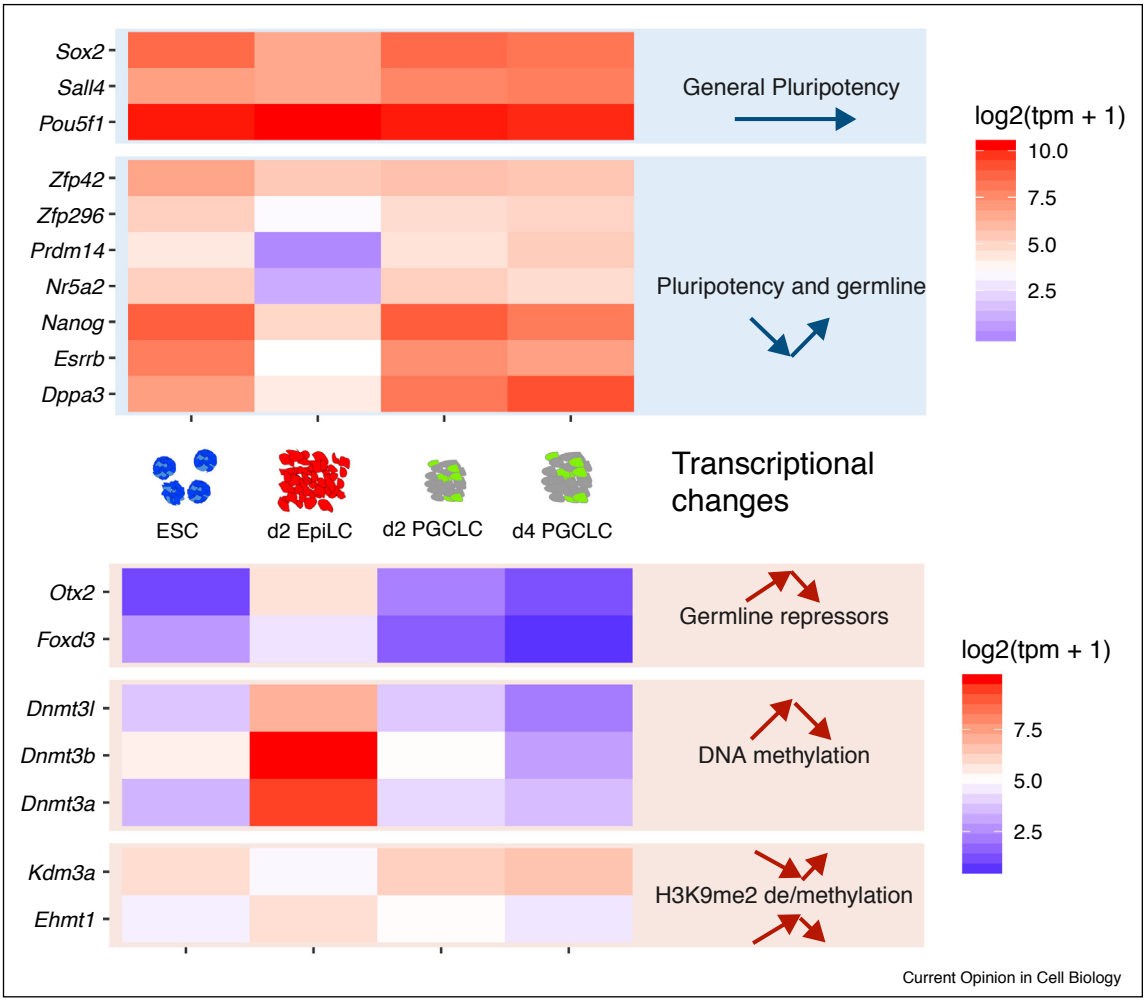
The developmental time frame of murine germline competence.
In vivo, primordial germ cells (PGCs) arise from epiblast cells from the proximo-posterior end of the ~E6.25 embryo. PGC specification can be recapitulated *in vitro* through the subsequent differentiation of embryonic stem cells (ESC) into Epiblast-like cells (EpiLC) and primordial germ cell like cells (PGCLC), while epiblast stem cells (EpiSC) are more similar to the post-gastrulation epiblast (E6.5). Overall, germline competence is restricted to the E5.5–E6.25 post-implantation epiblast cells *in vivo* and EpiLC *in vitro*, which are considered to display a 'formative' pluripotent state. In contrast, neither the 'naïve' (E4.5 epiblast *in vivo*; ESC *in vitro*) and 'primed' (E6.5 epiblast *in vivo*; EpiSC *in vitro*) pluripotent states nor the somatic lineages are competent for germline specification.

at relatively similar levels during these peri-implantation transitions (Figure 2) [9].

Exit from naïve pluripotency, establishment of a formative pluripotent state and acquisition of germline competence

The exit from the naïve pluripotent state is an asynchronous process that occurs *in vivo* upon implantation, between E4.5 and E5.5 [16••]. After implantation, ESC cannot be derived from epiblast cells, indicating a complete transition to a formative state [17]. Once established, the formative state is transcriptionally more uniform than the E4.5 or E6.5 epiblast [10•]. Recent analyses suggest that, with notable exceptions (e.g. PRDM14), the transcriptional silencing of naïve pluripotency genes in EpiLC is also reflected at the protein level [18]. Considering the poor germline competence of ESC [9], the previous observations suggest that PGCs do not arise from rare post-implantation cells in which the naïve expression program is retained. Instead, the complete dismantling of the naïve program seems to be a requirement for the acquisition of germline competence. Accordingly, it has been shown that upon exit from the naïve state, cells with low expression of *Zfp42/Rex1* (a naïve marker) resemble EpiLC and display higher germline competence than cells retaining high *Zfp42/Rex1* expression levels [18,19•]. Furthermore, the overexpression of the pluripotency/germline transcription factors *Nanog* [20] or *Prdm14* [21] in Day 2 EpiLC increases PGCLC specification, while earlier overexpression leads to a retention of an ESC-like state [21]. Although it is unclear why naïve pluripotency cells display reduced germline competence, work in ESCs suggests that JAK/STAT3 signalling diminishes germline competence and represses PGCLC formation through *Klf2* [22] and *Klf4* [23]. Hence, the acquisition of germline competence might entail repression of JAK/STAT3 signalling [24] to enable the reactivation of an expression program during PGC specification that resembles, but it is not identical (e.g. low *Klf4* expression) to the one present in naïve pluripotent cells [23].

Figure 2



Transcriptional changes of major regulators of mouse peri-implantation transitions.*
Pluripotency and germline genes with a reported relevance for germline specification are expressed in ESC and PGCLC but are repressed in the formative state (EpiLC), while the expression of general pluripotency genes is more constant.
In contrast, germline repressors, DNA and H3K9me2 methyltransferases (e.g. *Dnmt3b, Ehmt1*) are highly expressed in EpiLC but get silenced during PGCLC specification, while the H3K9me2 demethylases (e.g. *Kdm3a*) become upregulated.
*The RNA-seq data [48] were obtained from the European Nucleotide Archive, with accession number DRA003471.

It is important to emphasize that the extinction of the naïve pluripotency program is necessary but not sufficient for the acquisition of germline competence, since most naïve pluripotency genes are silenced not only in formative (E5.5–E6.5, EpiLC) but also in primed (>E6.5, EpiSC) epiblast cells, which are not germline competent [9]. Therefore, the establishment of a transient formative state is also required for germline competence. Accordingly, E5.5–E6.5 epiblast cells, regardless of their position within the embryo, and EpiLC display high germline competence when exposed to appropriate inductive signals (i.e. BMP4) [6,25]. Interestingly and despite their apparent transcriptional homogeneity, only a fraction (typically <20%) of E5.5–E6.5 epiblast cells or EpiLC give rise to PGCs even when exposed to high inductive signals [9]. As discussed in the following sections, these observations suggest that, epigenetic rather than transcriptional differences among formative cells and between formative and primed pluripotent cells might explain their distinct germline competence.

Despite the developmental relevance of the formative state [19•], we still have a limited understanding of the signals and transcription factors (TFs) that control its establishment. However, it has been recently shown that a dedicated network of transcription factors (e.g. *Foxd3*, *Otx2*, *Etv5*) coordinates both the silencing of naïve genes and the activation of early post-implantation epiblast markers. These TFs seem to preferentially operate in the context of enhancers, which undergo major reorganization during

the naïve to formative transition [15,26]. More specifically, FOXD3 acts as a repressor that binds and decommissions enhancers associated with pluripotency and germline genes, thus driving their silencing and promoting the exit from naïve pluripotency [27]. In contrast, OTX2 and ETV5 bind and activate multiple enhancers linked to early post implantation markers in order to implement the formative expression program [15,26]. Interestingly, although the previous TFs promote the transition towards formative pluripotency and, thus, the emergence of germline competence, these same TFs restrict the entry into the germline and they need to be silenced for PGC specification to proceed [27,28••]. Furthermore, the loss of *Otx2 in vitro* extends germline competence and enables PGCLC specification in the absence of extrinsic signals [28••]. Mechanistically, OTX2 might activate enhancers and genes specifically associated with formative pluripotency, including *Foxd3* and other repressors [15], which can then directly impair PGC specification by preventing the reactivation of pluripotency and germline genes [27].

PGC specification: reactivation of the naïve pluripotency expression programme

In response to extrinsic signals, formative epiblast cells at the proximo-posterior end of the E6.25 embryo initiate PGC specification. BMP4 emanating from the extraembryonic ectoderm represses OTX2, while WNT3 signalling from the visceral endoderm represses FOXD3, thus dismantling the formative program and preventing the acquisition of a somatic fate [28••,29]. Furthermore, WNT3 induces an incipient mesoendodermal program that, through the direct action of *T/Brachyury*, activates some of the earliest PGC specificers, *Prdm1* and *Prdm14* [7]. Almost concomitantly, many naïve pluripotency genes (e.g. *Nanog, Esrrb, Dppa3*) and additional PGC specifiers (e.g. *Tfap2c*) become reactivated (Figure 2), while the mesoendodermal genes get repressed by *Tfap2c* [30] and *Prdm1* [31,32]. The importance of reactivating the pluripotency program for PGC specification is illustrated by how the loss of pluripotency-associated TFs (e.g. *Nanog*, *Prdm14*, *Nr5a2*, *Esrrb*, *Zfp296*) leads to reduced PGC numbers and/or diminished fertility [33–36,37•]. Furthermore, pluripotency transcription factors such as *Nanog*, *Prdm14* or *Nr5a2* can induce PGC formation when overexpressed in EpiLC [20,21,37•]. Therefore, the reactivation of the naïve program is important for germline specification provided that pluripotency factors encounter an appropriate cellular (i.e. formative state) and signalling (i.e. BMP4, WNT3) context.

Epigenetic (re)programming of *cis*-regulatory elements during early PGC development

During peri-implantation development, epiblast cells undergo not only transcriptional but also epigenetic changes. With the exit of pluripotency, CpG methylation (mCpG) and H3K9me2 levels increase [12,14]. Then, the few epiblast cells located at the proximo-posterior end of the embryo that become PGCs revert this epigenetic program and, by E9.5, PGCs display global DNA hypomethylation, reduced H3K9me2/3 and increased H3K27me3 levels [38,39]. However, these major epigenomic reprogramming events occur once the initial steps of PGC specification, including the reactivation of the naïve pluripotency program, are already completed and thus, are unlikely to be causally involved in them. In this section we will focus on how changes in the epigenetic status of *cis*-regulatory elements (i.e. promoters and enhancers) might contribute to germline competence and early PGC specification.

DNA methylation

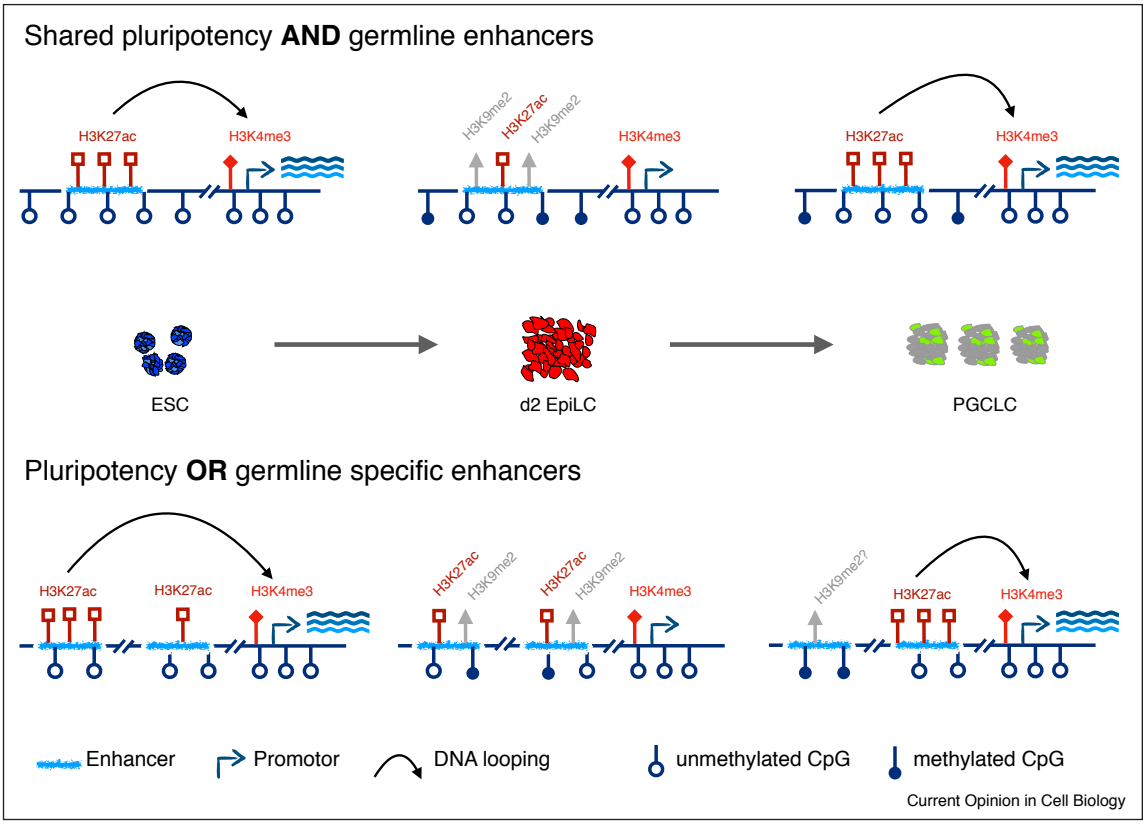
Once epiblast cells exit naïve pluripotency, global mCpG is initiated due to increased expression of *de novo* DNA methyltransferases (i.e. DNMT3A/B/L) (Figures 2 and 3) and de-repression of the methylation maintenance machinery (e.g. UHRF1) [12,14,40,41]. Single cell methylation data indicate that this increase in mCpG levels occurs in an oscillatory manner as epiblast cells acquire a formative state and gain germline competence. Thus, the formative state is characterized by low transcriptional variation yet high epigenetic heterogeneity [10•,11••]. This epigenetic heterogeneity is then progressively lost as epiblast cells progress towards a primed pluripotent state, characterized by global and uniform CpG hypermethylation [11••,13]. It is tempting to speculate that mCpG heterogeneity in the formative epiblast cells might be functionally connected with germline competence, with the cells that display an accessible genome, yet an inactive naïve expression program, preferentially acquiring a germline fate.

In contrast, during the transition from naïve to formative and primed pluripotency, not all genomic regions gain methylation with the same kinetics:

(i) despite their silencing, the promoter regions of most naïve pluripotency genes remain hypomethylated in formative and primed pluripotent cells [16••]. This is likely caused by the presence of CpG islands and the retention of H3K4me3 that together protect promoter regions from mCpG [42,43]. These observations suggest that promoter (de)methylation does not play a major role during the dismantling of the naïve expression program or its subsequent reactivation in PGCs.

(ii) naïve pluripotency enhancers gain mCpG as they get decommissioned upon transition into formative and primed pluripotency [16••]. Thus, mCpG methylation might be causally involved in the inactivation of the naïve expression program by directly acting on enhancers and contributing to their silencing. Furthermore, naïve enhancers in general and especially super enhancers display lower mCpG levels in the formative epiblast cells compared to primed ones

Figure 3



The reactivation of pluripotency genes in PGCs is regulated by enhancers.
Two scenarios illustrating how enhancers can control the reactivation of naïve pluripotency genes during PGC specification. Top: Some pluripotency genes are regulated by shared enhancers in ESC and PGCLC. The shared enhancers are active (H3K27ac) in both ESC and PGCLC but get decommissioned and become bivalent for H3K27ac and H3K9me2 in EpiLC [13,27]. Bottom: Other pluripotency genes are regulated by distinct enhancers in either ESC or PGCLC, which, in any case, are also inactive in EpiLC [32].

[16••], although it is currently unknown whether this has any influence on germline competence.

Active histone modifications

Active enhancers can be identified based on a unique chromatin signature that includes high levels of H3K27ac and H3K4me1 and low levels of H3K4me3 [44]. In accordance with the genome-wide profiling of these histone modifications, it has been shown that the developmental transitions leading to PGCLC specification involve major remodelling of enhancer landscapes while promoter regions remain comparably constant [32]. Despite the transcriptional similarities between naïve pluripotent cells and PGCs, these two cell types display considerably different enhancer repertoires, suggesting that, overall, the expression of the same set of genes is controlled by different enhancers in each cellular context [32]. However, it has also been noted that a set of enhancers preferentially associated with key naïve pluripotency and early PGC regulators (e.g. *Prdm14*, *Prdm1*, *Tfap2c*) might be active in both ESCs and PGCLCs (Figure 3) [27]. During the transition from naïve to formative pluripotency, these enhancers get decommissioned by losing H3K27ac but retaining H3K4me1 [27], which could prime their subsequent activation in PGCs [45]. Interestingly, some of these enhancers display rather unique chromatin features in ESC, as they are bound by MLL2/KMT2B and enriched in H3K4me3, two proteins that are typically observed within promoters [46••]. Moreover, the loss of MLL2/KMT2B in ESC compromises PGC specification, presumably due to its regulatory function within enhancers controlling the expression of PGC specifiers (e.g *Prdm14*) [46••]. Overall, the current evidences suggest that changes in active histone modifications and/or enzymes mediating their deposition within enhancers might be important for both germline competence and PGC specification.

Repressive histone modifications

Similarly to the global increase in mCpG during the transition from naïve to formative and primed pluripotency, H3K9me2 also increases during these developmental

transitions [13]. Furthermore, H3K9me2 is dramatically reduced once PGC start migrating (>E7.5), concomitantly with silencing of H3K9 methyltransferases (*Ehmt1/2*) and activation of H3K9 demethylases (*Kdm3a/b*) [39,47]. Changes in H3K9me2 within promoters and/or enhancers might be causally involved in the repression of a subset of naïve pluripotency and germline genes as epiblast cells exit pluripotency and, thus, could contribute to the proper acquisition of germline competence. In agreement with this possibility, mutations in genes that globally influence H3K9me2 profiles result in germline defects [33,37•]. During the enhancer decommissioning that accompanies the transition from naïve to formative pluripotency, certain enhancers associated with naïve/germline genes are transiently enriched in both H3K27ac and H3K9me2 [13]. This enhancer bivalency might facilitate the reactivation (H3K27ac gain; H3K9me2 loss) of some enhancers and their target genes during early PGC specification (Figure 3) [32,48]. In contrast to the dramatic changes in H3K9me2 levels that occur during PGC development, H3K9me3 and its associated methyltransferases (*Suv39h1/2, Setdb1)* and demethylases (*Kdm4a/b/c)* remain more stable [39,47]. Nevertheless, *Setdb1* mediated H3K9me3 is required for PGC specification, presumably through the repression of *Otx2* [49].

The reprogramming of H3K9me2 and mCpG genomic distribution that occurs during mouse peri-implantation transitions might be explained, at least partly, by metabolic changes. ESCs exhibit an oxidative metabolism that switches towards glycolysis in EpiLC. Subsequently and as PGCLC specification starts, a preferential oxidative metabolism is re-established. As a result, both ESC and PGCLC display higher alpha-ketoglutarate levels than EpiLC and, notably, the addition of alpha-ketoglutarate can extend the time-frame of germline competence [50•]. Since alpha-ketoglutarate is an essential cofactor for several enzymes involved in H3K9me2 (KDM3A/B) and mCpG (TET1/2) demethylation, oxidative metabolism might contribute to the global depletion of H3K9me2 and mCpG in naïve pluripotency and PGCs. Taken together, as naïve epiblast cells transit to formative and primed pluripotency, the expression of H3K9me2 (*Ehmt1/2*) and mCpG methyltransferases (*Dnmt3a/b/l*) increases while the activity of H3K9me2 and mCpG demethylases decreases. Overall, this results in a global genomic heterochromatinization that could progressively restrict germline competence and favor somatic cell fates.

Future work should aim at elucidating the causal roles and mechanisms whereby epigenetic regulators and their associated modifications contribute to germline competence and PGC specification. This could potentially facilitate the use of epigenetic drugs to modulate these processes and, thus, improve the *in vitro* generation of germ cells with the ultimate goal of treating human infertility.

Conflict of interest statement

Nothing declared.

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