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Conceptual links between DNA methylation reprogramming in the early embryo and primordial germ cells

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DNA methylation is a carrier of important regulatory information that undergoes global reprogramming in the mammalian germ line, including pre-implantation embryos and primordial germ cells (PGCs). A flurry of recent studies have employed technical advances to generate global profiles of methylation and hydroxymethylation in these cells, unravelling the dynamics of methylation erasure at single locus resolution. Active demethylation in the zygote, involving extensive oxidation, is followed by passive loss over early cell divisions. Certain gamete-contributed methylation marks appear to have evolved non-canonical mechanisms for targeted maintenance of methylation in the face of these processes. These protected sequences include the imprinting control regions (ICRs) required for parental imprinting but also a surprising number of other regions. Such targeted maintenance mechanisms may also operate at certain sequences during early PGC migration when global passive demethylation occurs. In later gonadal PGCs, imprints must be reset and this may be achieved through the targeting of active mechanisms including oxidation. Thus, emerging evidence paints a complex picture whereby active and passive demethylation pathways operate synergistically and in parallel to ensure robust erasure in the early embryo and PGCs.

Addresses

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Introduction

Epigenetic reprogramming takes place in the germ line of animals and plants, and involves major remodelling of transcription, histone modifications and histone variants, and DNA methylation. This reprogramming has wide ranging implications for development, disease,

transgenerational epigenetic inheritance, and ageing. In mammalian development, two major waves of reprogramming reset the epigenome: the first wave occurs following fertilization in the early embryo and the second takes place in primordial germ cells (PGCs) which are the embryonic progenitors of sperm or oocytes [1,2]. In both cases, epigenetic reprogramming includes the global erasure of DNA methylation marks followed by extensive remethylation. Cells in which DNA methylation reprogramming occurs can only be obtained in small numbers, imposing a significant technical challenge for a detailed understanding of epigenetic reprogramming through molecular work.

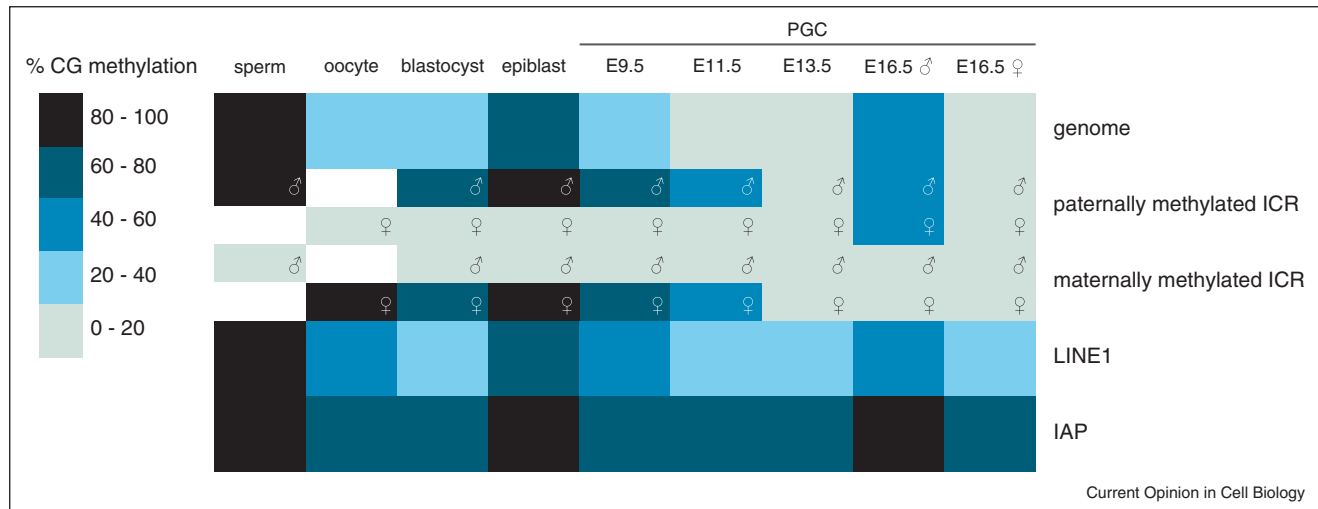
The last few years have seen key technical advances that have allowed a number of laboratories to generate the first genome-wide high-resolution methylation profiles of early embryonic samples and PGCs at crucial time points during the reprogramming process [3,4,5^{••},6[•],7,8^{••},9,10^{••},11^{••},12,13]. These studies permit a detailed dissection of DNA demethylation dynamics during epigenetic reprogramming. In addition, a number of studies have investigated the role of several factors that play a role in DNA methylation erasure in the early embryo and PGCs, further adding to our mechanistic understanding of DNA demethylation [3,10^{••},11^{••},14,15[•],16,17[•],18[•],19^{••},20].

The dynamics of DNA methylation reprogramming in the early embryo

Global DNA methylation levels differ significantly between male and female gametes with 40% in oocyte and 90% in sperm at fertilization [6[•]] (Figure 1). The parental genomes united at fertilization thus make highly disparate epigenetic contributions to the new embryo. Sperm-specific methylation is largely restricted to repetitive and intergenic sequences in line with its global hypermethylation, with relatively few methylated CpG islands (CGIs, see Box 1) [5^{••},6[•],8^{••}]. In contrast, the oocyte contributes a significant number of this latter class, with over 1300 CGIs differentially methylated between oocyte and sperm [4,5^{••},6[•]].

Over a decade ago, pioneering studies demonstrated a global and active loss of methylation from the paternal genome but not its maternal counterpart, which is instead passively demethylated (see Box 1 for definitions) during the following cleavage divisions [21–23]. This wave of zygotic erasure does not affect all regions of the paternal genome equally. The repetitive elements forming the bulk of the sperm's unique methylation contribution are a well-established target [15[•],24], but recent comprehensive

Figure 1



Methylation heatmap of various genomic regions during embryonic development. Methylation is actively removed from the globally hypermethylated paternal genome after it unites with the hypomethylated oocyte genome at fertilization. Passive loss over early cell divisions lowers methylation levels in the new embryo before a wave of *de novo* methylation begins around implantation. Another round of global erasure ensues in developing PGCs, with subsequent remethylation occurring from around E14.5 in male cells, and postnatally in oocytes. Imprint control regions (ICRs) arrive in the zygote with gamete-specific methylation that is maintained throughout development until reprogramming between E11.5 and E13.5 in PGCs. The next generation of imprints is laid down concomitant with global remethylation. While the long interspersed element 1 (LINE1) retrotransposons undergo similar methylation reprogramming to the overall genome, intracisternal A particle (IAP) retrotransposons maintain a high level of methylation throughout development.

profiling reveals significant variation in the degree of demethylation across different element classes, and even within their component families [8^{••}]. These differences may reflect the need to ensure correct transcriptional activation in the early embryo while maintaining repression of potentially dangerous retrotransposition activity. Other regions have a more evident requirement for maintenance of methylation in the face of global erasure — such as the imprinting control regions (ICRs) crucial to parental imprinting, which are protected against both active demethylation in the zygote and the ensuing passive loss [6[•],25] (Figure 1).

Methylation profiling of the hypomethylated blastocyst led to the surprising finding that ICRs are not the only regions to resist DNA methylation erasure in the early embryo: the majority of oocyte-specific CGIs along with a subset of sperm-specific CGIs retain higher than predicted methylation [4,5^{••},6[•],8^{••}]. In addition, repetitive elements such as the intracisternal A particles (IAPs) class (the most recent and still potentially active retrotransposons in the rodent genome), which are highly methylated in both sperm and oocyte appear to be almost completely resistant to demethylation in the early embryo [6[•],8^{••},24] (Figure 1). This is an important insight into the longevity of these methylation marks and demonstrates that gametic methylation is a key driver of methylation fate in the early embryo.

Thereafter, remethylation takes place rapidly in the transition from blastocyst to epiblast reaching around 70% methylation by E6.5 [4,8^{••},10^{••}]. *De novo* methylation in the epiblast targets promoters of various lineage-specific genes such as those involved in gametogenesis and hematopoietic development [4]. For regions that must maintain parent-specific methylation, such as ICRs, protection of the unmethylated allele from this wave of *de novo* methylation is instrumental [9].

The dynamics of DNA methylation reprogramming in PGCs

The remethylated epiblast is the birthplace of PGCs, which are thought to inherit the newly established DNA methylation pattern from epiblast cells [10^{••}].

A number of recent studies suggest that DNA methylation erasure in PGCs occurs at two stages during their development: the first one coincides with the migration phase from around E8.5 and the second with the gonadal stage from around E10.5 [7,10^{••},11^{••},19^{••},26]. DNA methylation erasure during the migration phase is truly global affecting almost all genomic features [10^{••}]. However, a number of regions become demethylated with slower kinetics than the rest of the genome: As in the early embryo this includes not only ICRs but also CGI promoters of germ cell specific and meiosis related genes, and CGIs associated with the inactive X chromosome;

Box 1 Glossary

5-Methylcytosine (5mC)	Cytosine with a methyl group on the fifth carbon, predominantly found in CpG context in mammalian DNA.
Epigenetic reprogramming	Resetting of the previously existing epigenetic landscape that includes erasure of DNA methylation marks followed by extensive remethylation. Other epigenetic marks such as histone modifications and histone variants are also reprogrammed.
DNA methyltransferases (<i>Dnmts</i>)	There are four family members; <i>Dnmt1</i> is the canonical maintenance methyltransferase, <i>Dnmt3a</i> and <i>Dnmt3b</i> are <i>de novo</i> methyltransferases (but can also be involved in methylation maintenance), and <i>Dnmt3L</i> is a non-catalytic orthologue involved in recruiting <i>Dnmt3a</i> and <i>Dnmt3b</i> to sites of <i>de novo</i> methylation.
Methylation maintenance	At the replication fork, methyl groups on the parental strand are recognized by nuclear protein 95 (Np95 or Uhrf1) and copied onto the newly synthesized strand by <i>Dnmt1</i> .
<i>De novo</i> methylation	Addition of methyl groups to previously unmodified cytosine by <i>Dnmt3a</i> or <i>Dnmt3b</i> .
Passive demethylation	Progressive dilution of 5mC or its oxidized derivatives by a lack of maintenance at DNA replication. This can be achieved by the exclusion from the nucleus of proteins required for maintenance methylation, such as <i>Dnmt1</i> and Np95.
Active demethylation	Removal of 5mC not based on a diluting effect during DNA replication. This is not to imply that active removal is necessarily independent of DNA replication, as it may require the molecular environment, such as signalling events, that replication invokes. Proposed active mechanisms include oxidation by Tet proteins, and entrance into the BER pathway (see below).
Targeted methylation maintenance	Methylation maintenance of specific sequences during passive demethylation. In the early embryo, Zinc finger protein 57 (Zfp57) recognizes methylation at ICRs and recruits together with Krüppel associated protein 1 (Kap1 or Trim28) proteins of the <i>Dnmt</i> family to maintain methylation at these sequences.
Ten-eleven-translocation proteins (Tets)	A family of three oxidases (Tet1, Tet2 and Tet3) catalyzing the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).
Base excision repair (BER)	The cellular system responsible for resolution of small DNA lesions by the excision of affected bases and replacement with newly synthesized DNA. Components of this pathway can act on derivatives of 5mC to orchestrate active demethylation through its replacement with unmodified cytosine. Notably, thymine-DNA-glycosylase (Tdg) can excise thymine (generated by the deamination of 5mC by Activation induced deaminase [Aid]), 5fC and 5caC (generated by oxidation of 5mC by Tet enzymes — see above), and 5hmU (a possible deamination product of 5hmC) to initiate BER. Thus, oxidation of 5mC can promote entry into the BER pathway but this can also occur independently through the direct deamination of 5mC.
Imprinting control region (ICR)	DNA sequence with differentially methylated alleles, which controls the monoallelic expression of a cluster of genes depending on their parental origin. The methylation marks at ICRs are established in the germ line of the parents in a gender-specific manner.
CpG island (CGI)	CpGs are generally methylated in mammalian genomes and are therefore depleted due to the mutagenic properties of 5mC. However, short regions of DNA (on average 1000 bp) known as CGIs contain elevated CpG density. These are generally sites of transcription initiation; around 70% of annotated gene promoters are associated with a CGI. Promoter CGIs are typically hypomethylated, with important exceptions including regions with parent-specific methylation such as ICRs, and some developmental promoters that are silenced in differentiated cells [51].

methylation at these regions is only lost completely in the second demethylation phase from E11.5 [7,10^{••},11^{••},19^{••}]. This is in line with previous reports describing methylation erasure at ICRs and promoters of germ line specific genes from E11.5 [27–31]. DNA methylation erasure in PGCs is completed in the gonadal stage and results in a globally hypomethylated state at E13.5 [1,2].

Few regions escape DNA methylation erasure in PGCs and these mostly include IAPs. Other repetitive elements such as the long interspersed element 1 (LINE1) and short interspersed element (SINE) groups are largely reprogrammed; these contrasting dynamics mirror the complex demethylation patterns of retrotransposons in the zygote [7,10^{••},24]. A number of studies have identified regions that escape methylation erasure in PGCs and there seems to be a positive correlation between likelihood of resistance and proximity to an IAP [7,10^{••},11^{••}]. However there is also a limited number (a couple of hundred) of CGIs not linked to IAPs, which show variable resistance to reprogramming and may thus contribute to transgenerational epigenetic inheritance [10^{••},11^{••}].

Methylation marks are re-established in male PGCs by E16.5 reaching about 50% global methylation levels while female PGCs maintain the hypomethylated state from E13.5 to E16.5 [10^{••}] (Figure 1). *De novo* methylation in female germ cells takes place in growing oocytes restoring methylation levels to the final methylation levels of about 40% characteristic for oocytes [5^{••},6[•]]. This means that further *de novo* methylation has to take place in male PGCs *en route* to reaching the high methylation levels in sperm and it is unclear at this point if there is exclusively further *de novo* methylation or if additional DNA demethylation and therefore methylation reprogramming takes place during male germ cell development.

Mechanisms for DNA methylation reprogramming

Global erasure

DNA methylation can be erased through active or passive mechanisms, or by a combination of the two (see Box 1). The gradual loss of methylation in the early embryo is the result of a passive mechanism owing to the predominant exclusion of *Dnmt1* [32] and Np95 (F Santos, M Oda, W Dean, personal communication) from the nucleus of early

embryonic cells. While this accounts for the loss of methylation contributed by the oocyte, active mechanisms also act to remove methylation from the paternal genome in the zygote [as described above]. Both the elongator complex and the base excision repair (BER) pathway (see Box 1) have been implicated in this process [14,33], but their precise role has yet to be dissected. Recent work has uncovered that Tet3 plays a crucial role in active erasure by oxidizing 5mC to 5hmC, 5fC and 5caC in the zygote (see Box 1). These oxidized derivatives can then be lost passively over the following cell divisions concomitant with maternal 5mC [34,35], or provide a substrate for further enzymatic activity leading to unmodified cytosine — such as entrance into the BER pathway [36–38]. It is surprising that oxidation should be needed to demethylate the paternal genome given the subsequent passive loss over cleavage divisions. Perhaps certain sequences require early demethylation in the zygote for transcriptional activation; alternatively 5hmC, 5fC and 5caC may constitute epigenetic signals with as yet unknown roles in the early embryo. In either case, the emerging evidence supports a scenario whereby active and passive mechanisms act in concert to achieve global methylation erasure in the early embryo.

Recent evidence suggests that passive demethylation is also the basis for global methylation erasure in PGCs: methylation levels are gradually reduced correlating with the increase in cell numbers, *Np95* is transcriptionally downregulated and the remaining protein seems to be excluded from the nucleus, and *Dnmt1* seems to be excluded from replication foci [7,10,11,19]. Furthermore, PGCs of E9.5 show high numbers of hemimethylated CG sites, which arise when DNA methylation maintenance is impaired [10].

Active DNA demethylation pathways including *Aid* and *Tdg* (see Box 1, BER entry) have been shown to contribute to methylation erasure in PGCs [3,16]; the BER pathway has also been implicated by the same study that demonstrated its involvement in zygotic demethylation [33]. In addition, evidence for oxidative removal of 5mC through Tet1 and Tet2 has been provided [11,12], but methylation levels at the potential time points for 5mC conversion are already low [10] and methylation levels of E13.5 PGCs in *Tet1* mutants are only marginally affected [18]. It may be that global oxidative removal of 5mC occurs earlier than previously anticipated at time points that have so far not been profiled for their methylation levels (E7.5–E8.5) and lack thereof could be compensated for by passive demethylation. However, *in vitro* PGC derivation from ES cells lacking Tet1 and Tet2 seems to be unaffected, making a role for the Tet proteins in global methylation erasure in PGCs an unlikely proposal [20]. Alternatively, it has been suggested that oxidative removal of 5mC could be a locus-specific phenomenon rather than a global one [12,20]. In such a

scenario, global methylation erasure for the bulk of the genome would occur in early PGCs (E8.5–E10.5) largely by a passive mechanism. Remaining methylation at sequences that demethylate late in PGC development such as ICRs would then be removed from E10.5 by the oxidative activity of the Tet proteins. In line with the latter suggestion, a targeted role for oxidative removal of 5mC at ICRs and promoters of meiosis specific genes has been described *in vivo* and *in vitro* [11,18,20,39]. In addition, antibody staining suggests that lack of Tet1 and Tet2 does not affect global erasure in PGCs [40]; however some progeny of *Tet1* and *Tet2* double knockout mice show imprinting defects, indicating that the oxidative pathway is required for complete imprint erasure during PGC development [43]. Further molecular evidence is needed to deepen our understanding of the role of the Tet proteins in methylation erasure in PGCs but it seems that — as in the early embryo — active and passive mechanisms work in parallel to achieve global methylation erasure.

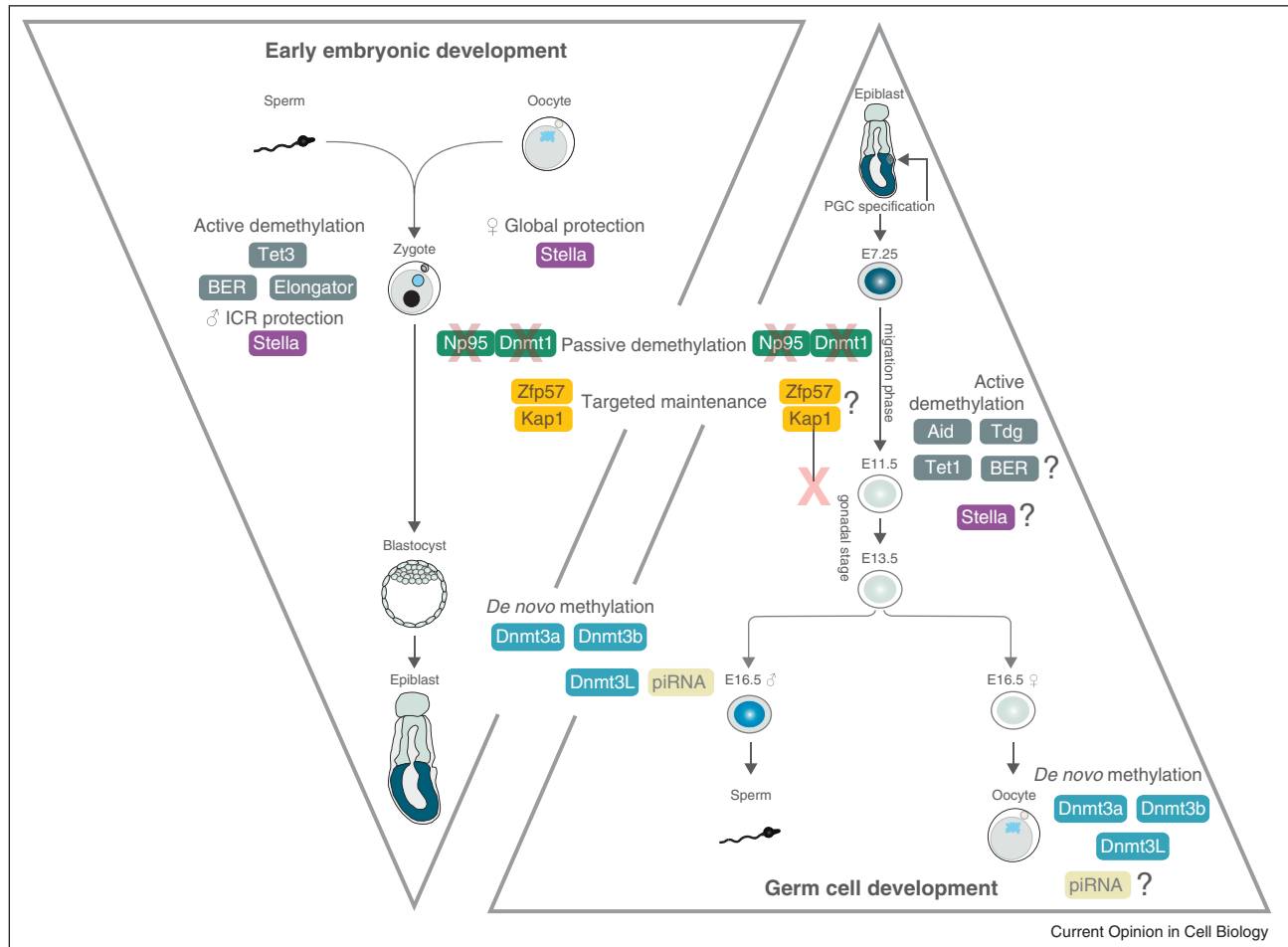
Protection against demethylation and targeted maintenance

DNA methylation reprogramming is a genome-wide phenomenon, however, certain regions are protected against demethylation. IAPs make up the sequence class that seems most highly protected against demethylation in the zygote, the early embryo, and in PGCs. The fact that IAPs are consistently protected suggests the presence of a universal mechanism. IAPs fail to attract 5hmC and are highly dependent on *Dnmt1* and *Np95* for methylation maintenance [41,42] but may also use non-canonical targeting mechanisms for *Dnmt1*.

In the zygote, the maternal factor *Stella* (aka *Dpp3a* or *Pgc7*) is essential for the protection of 5mC in the maternal genome as well as at paternally methylated ICRs [43]. *Stella* exerts its protective effect through the inhibition of Tet3 binding, thereby preventing oxidation of 5mC [44]. Imprints are also maintained during the subsequent passive demethylation in the early embryo [25]. In this case, *Zfp57* and *Kap1* (or *Trim28*) have been shown to recruit proteins of the *Dnmt* family to ICRs and maintain methylation at these sites during passive demethylation [45,46].

ICRs and the CGI promoters of gametogenesis-related genes (as well as CGIs on the X-chromosome) are maintained at high methylation levels during passive DNA demethylation in PGCs until around E11.5, at which point these regions undergo full demethylation [7,10]. The delayed demethylation pattern of these regions until E11.5 is highly reminiscent of that in the early embryo and it has been suggested that *Zfp57* may also be involved in methylation maintenance of these regions in PGCs [10] (Figure 2). In addition, the protection factor *Stella* is highly expressed in PGCs but it is unclear whether it plays a role in protecting methylation marks. Additional *in*

Figure 2



Mechanistic links between DNA methylation reprogramming in the early embryo (left triangle) and in primordial germ cells (right triangle). Passive loss of methylation by exclusion of Dnmt1 and Np95 (see Box 1) from the nucleus is a key feature of reprogramming in both pre-implantation embryos, where it occurs over early cleavage divisions, and in PGCs during the migration phase. In each case, methylation at certain regions such as ICRs (see Box 1) is maintained – in the early embryo this protection is dependent on Zfp57 and Kap1; this machinery may also operate to confer protection in PGCs. Passive demethylation in the early embryo is preceded by a wave of active removal from the paternal genome in the zygote, which involves the elongator complex, the base excision repair pathway, and oxidation by Tet3. Some of these activities may also be targeted to the sequences protected against passive demethylation in PGCs when they must be reprogrammed. A phase of *de novo* methylation by Dnmt3 proteins (see Box 1) follows erasure in the early embryo and PGCs, the latter case occurring earlier in male than in female cells.

in vivo analysis is needed to study the role of these factors in targeted methylation maintenance in PGCs, however it seems that before the gonadal stage of erasure the dynamics of demethylation and maintenance of specific regions share striking similarities with those in the early embryo.

Methylation erasure at sequences with targeted maintenance

The recent wealth of molecular data describing DNA methylation reprogramming in the zygote, the early embryo, and in PGCs has put passive demethylation into the spotlight for global methylation erasure. However, certain regions in the genome that carry long-term functional methylation marks such as ICRs and promoters of

germ line specific genes appear to have evolved a non-canonical maintenance mechanism (involving Zfp57 and Kap1 and perhaps others) that is able to operate when the canonical maintenance pathway (involving Np95) is impaired. This means that removal of these methylation marks has to rely on other mechanisms than passive demethylation alone.

ICRs become demethylated in PGCs around E10.5, which is the time point at which ICRs acquire 5hmC [11^{••}]. Also, promoters of germ line specific genes have similar demethylation kinetics, also acquire 5hmC, and these genes are misregulated in *Tet1* KO PGCs indicating a role for hydroxylation of these specific methylation marks [10^{••},18[•],30,31]. Perhaps 5hmC is not recognized

by the non-canonical maintenance mechanism and thus, ICRs and promoters of germ line specific genes become sensitive to passive demethylation upon hydroxymethylation from around E10.5 [19^{••}]. Indeed *Zfp57* prefers to bind to its target sequence when methylated, but not when hydroxymethylated [47], providing a potential mechanism for such a switch.

It seems paradoxical that certain methylation marks are maintained in migrating PGCs if they are destined to be erased in gonadal PGCs. It is possible that this paradox is simply a consequence of the non-canonical methylation maintenance mechanism that these regions have evolved, and which seems to be universally in place in early PGCs, ESCs, and cells of the early embryo to ensure robust maintenance even when global methylation erasure occurs. In PGCs, where imprints have to be reset and promoters of germ line specific genes have to be demethylated, this mechanism is then impaired from E10.5 leading to the final demethylation of these sequences in gonadal PGCs.

Outlook

The picture that emerges from the body of recent data is complex; different mechanisms have evolved for the maintenance of methylation at specific sequences, as well as for its removal. This erasure programme involves both active and passive processes and significant functional redundancy which, while necessary to ensure robust demethylation, complicates mechanistic analysis of individual pathways. It will be important in future work to dissect how the various means for methylation erasure are linked and integrated, and how they are regulated by signalling pathways in the germ line and the early embryo.

Genome-wide epigenetic reprogramming in the germ line is possibly an adaptation which is specific to mammals, since evidence for such a mechanism in other vertebrates, non-vertebrates or seed plants is lacking [48]. Perhaps the relatively late allocation of the mammalian germ cell lineage (after epigenetic priming has occurred for the embryonic lineages) necessitates extensive reprogramming for the epigenetic ground state of pluripotency, immortality of the germ line, and to avoid transgenerational epigenetic inheritance on a large scale. An essential outcome of reprogramming is the erasure of imprints in PGCs, and in this context it is noteworthy that seed plants have imprinting but this is primarily limited to the endosperm (the plant equivalent of the placenta) and is achieved by demethylation of imprinted loci in this tissue [49]. Hence there is no need in plants for imprint erasure in the germ line, and global methylation erasure is apparently absent in plant germ cells.

Different logic must be applied to describe the evolutionary forces shaping reprogramming in the early embryo, where imprints must instead be maintained. Here, global

methylation remodelling may be required for the switch from germ cell programmes to the totipotent state of the new embryo, as well as ensuring any parental epimutations are not perpetuated. Analogous to plants, mammalian genomes may also erase methylation at repetitive elements to allow their expression and therefore detection by the cellular systems, such as piRNAs that subsequently orchestrate their long-term repression [50]. Interestingly, while the demethylation of the paternal genome in the zygote may contribute to these effects, its active nature — in contrast with the passive loss over cleavage divisions — may hint at an attempt by the oocyte to remove paternal imprints for maternal benefit, invoking a ‘battle of the sexes’ scenario. Passive demethylation in the early embryo appears to mirror global erasure in PGCs in form and function, while the removal of methylation at imprinted regions seems to be kinetically and mechanistically distinct. This comparison hints at two discrete ‘modes’ of methylation reprogramming: one associated with the restoration of developmental potency — occurring in both the early embryo and PGCs — and one required to reset imprints for the next generation, restricted to PGCs.

Key for future work will be to separately address the mechanisms involved in imprint reprogramming and global reprogramming in order to fully understand the biological implications for epigenetic reprogramming in the mammalian germ line.

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