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## Point of View

# Epigenetic reprogramming in mammalian reproduction

## Contribution from histone variants

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Development of the mammalian embryo is, by definition, epigenetic. At the level of the nucleosome, the building block of the chromatin, changes in chromatin structure can be regulated through histone content. Apart from the canonical histones whose synthesis is restricted to S-phase, different histone variants have been identified. Histone variants can help to establish specialised chromatin regions and to regulate developmental and cell differentiation processes. While the role of histone variants has been extensively explored in differentiated cells, less is known in germ cells and embryos. Increasing lines of evidence suggest that the functions and/or properties of histone variants in embryos might be different to those in somatic cells. During reprogramming, histone variants such as H3.3 or H2A.Z are candidates to play potential important roles. We suggest that H3.3 has an important role in setting up a 'transition' signature, and provides the possibility to infer changes in chromatin architecture independent of DNA replication. This should confer flexibility during important developmental processes. The specific pathways through which H3.3 could regulate different chromatin conformations at different loci and the identification of specific proteins responsible for this deposition are an important challenge for future investigation. Lastly, the set of variants incorporated within the nucleosome can have important consequences in the regulation of epigenetic mechanisms during development.

Eukaryotic gene expression is deeply influenced by changes in chromatin structure. When these changes are independent from the DNA sequence per se and are inherited through cell division, they can constitute epigenetic signatures. Apart from DNA methylation, most often these changes occur at the level of the building block of the chromatin, the nucleosome. The nucleosome is formed by two copies of each of the core histones: H3, H4, H2A and H2B, around which a ~146 bp piece of DNA is wrapped. The so called canonical histones are incorporated into the chromatin exclusively

during S-phase, their expression is cell cycle regulated and they are transcribed from multiple genes often distributed in clusters.<sup>1-4</sup> However, for all histones—apart from H4—alternative histone variants exist. These histone variants are also expressed and incorporated outside of S-phase and are transcribed from single-copy genes.<sup>5-7</sup> Importantly, some of these variants have acquired specialised functions in developmental processes such as fertilization and X-chromosome inactivation. Here we discuss and present some hypotheses on the contribution of two types of variants: those of H3 and of H2A as well as their potential role in directing epigenetic information in mammalian development.

### Histone H3 Variants: Small Differences Can be Big

Most studies concerning histone variants have focused on variants of H3. In mammals, there are two canonical histones of H3: H3.1 and H3.2 as well as three other H3 variants: H3.3, a testis specific H3 variant (H3t) and centromeric H3 variants (CenH3s). We will focus on the three variants H3.1, H3.2 and H3.3, as they have been studied throughout key developmental processes and in cell differentiation systems, including mouse embryos and ES cells. Only one amino acid differs between H3.1 and H3.2, located at position 96 (a Cys-Ser substitution) and five amino acids are different between H3.1 and H3.3 at positions 31, 87, 89, 90 and 96 (Ala-Ser, Ser-Ala, Val-Ile, Met-Gly, Cys-Ser substitutions respectively).<sup>8</sup> The differences in amino acid sequence amongst them, albeit surprisingly small, lead to different pathways of incorporation into the chromatin for H3.1/H3.2 on the one hand and H3.3 on the other hand.<sup>5,9,10</sup> In somatic cells, it has been well established that the canonical variants H3.1 and H3.2 are synthesised only during S-phase and then deposited into the chromatin through a Replication Coupled (RC) pathway.<sup>9,11,12</sup> Recently, it was further shown that these two variants can also be incorporated in the context of DNA repair.<sup>13</sup> More generally, the canonical variants of H3 are deposited into chromatin concomitant and/or in a pathway that involves DNA synthesis. On the contrary, H3.3 is synthesised throughout the cell cycle and is incorporated into chromatin by a Replication Independent (RI) pathway, hence its name of 'replacement variant.'

As a general rule, H3 is deposited as H3-H4 tetramers.<sup>10-12</sup> RI and RC deposition of H3 into the chromatin are carried out by different histone chaperones. In HeLa cells, the RC deposition complex for H3.1/2 contains the histone chaperone ASF-1 and the entire CAF-1 complex, while the RI deposition complex of H3.3

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contains ASF-1, the smallest subunit of the CAF-1 complex (p48) and a specific histone chaperone HIRA.<sup>10,14</sup> CAF-1 can interact with PCNA and promote subsequent deposition of H3.1/2-H4 heterodimers to sites of DNA synthesis, allowing for the assembly of new nucleosomes at the replication fork.<sup>15</sup> The absence of deposition of H3.1/2 outside of the S-phase cannot be explained exclusively by the fact that these variants are not available during the entire cell cycle. Indeed, in *Drosophila*, the pathway of assembly for H3 canonical variants is specified by the residues located in positions 87, 89 and 90, which differ among H3.1/H3.2 and H3.3 variants.<sup>5,9</sup>

Once incorporated into nucleosomes, H3.1, H3.2 and H3.3 will have different effects on chromatin-mediated processes. Indeed, studies in mammalian cells show that upon transcriptional induction, H3.3 is recruited at highly expressed loci,<sup>16-18</sup> while H3.1 and H3.2 are potentially linked with both active and inactive genes. Despite their high degree of similarity, mass-spectrometric analysis of histone H3 variants has revealed that the post-translational modifications (PTMs) occurring on the three variants differ: H3.3 is mostly associated with marks of transcriptionally active chromatin, H3.2 is enriched in repressive marks and H3.1 contains PTMs of active and inactive chromatin.<sup>19-21</sup> It is important to note that, to date, this is the only evidence that H3.1 and H3.2 could have different functions. It has also been shown that when H3.3 is present in the chromatin, the surrounding nucleosomes, even if containing H3.1, will have predominantly marks of active transcription.<sup>22</sup> So, it appears that, in somatic cells, H3.1 and H3.2 are deposited rather broadly on the genome during replication and could play roles both in active or inactive genomic regions, most likely due to their content of different PTMs. On the contrary, deposition of H3.3 could be more regulated, and perhaps targeted to specific genomic regions, as it is the case of sites of active transcription.

There are further considerations for H3.3 deposition outside of S-phase. It has been suggested that the deposition of H3.3 at transcribed genes is the result of a neutral replacement, in a replication-independent manner, of nucleosomes that have been evicted by the transcription machinery.<sup>23</sup> However, other studies suggest that H3.3 by itself, even in the absence of post-translational modifications, could act as a marker for active transcription.<sup>16</sup> These conclusions are based on findings that show conservation of H3.3 on the chromatin within a highly active transgene through mitosis, which is expected to promote gene activation immediately after exit from mitosis.<sup>16</sup> However, the pathways responsible for controlling specific deposition of H3.3 to active genes and the pathways of recognition of H3.3 by the transcription machinery still have to be elucidated. An important feature is that nucleosomes containing H3.3 appear to be less stable than those containing H3.1 or H3.2,<sup>24</sup> and could therefore be more easily removed, thereby allowing transcription and/or local chromatin remodelling.

### H3 Variants in Reprogramming During Mammalian Development

The roles played by the different variants of H3 in germ cells and during preimplantation development are less well described compared to what is known in somatic cells, most likely due to the technical limitations of both systems. The current lack of antibodies specific for the different variants does not facilitate this task. However, recent works have begun to highlight the fact that H3 variants could have

roles in sexual reproduction that are different from their roles in somatic cells. Whether the three H3 variants display a similar pattern of PTMs in embryos and in somatic cells is not known, but most likely the repertoire of modifications in each of the three variants differ in the two situations. For example, we have previously reported a dicotomy between H3.3 enrichment in the male pronucleus and a lack of the active marks H3K4me2 and H3K4me3 following fertilization.<sup>25</sup>

In germ cells and embryos, two major events of large-scale replacement of chromatin marks (DNA methylation, histone PTMs, histone content) occur. In mouse primordial germ cells (PGCs), parental imprints are erased and re-established in a sex-specific manner. Monoallelic parent-of-origin specific expression of imprinted genes is regulated by epigenetic modifications. The reprogramming of PGCs occurs between embryonic day (E) 11.5 and E12.5. During this reprogramming event, it has been inferred, because of the transient presence of HIRA in the nucleus of the PGCs between E11.5 and E12.5, that H3.3 could play a role in this phenomenon.<sup>26</sup> Another event involving huge reprogramming occurs immediately following fertilization when two highly differentiated cells, the oocyte and the sperm, give rise to a totipotent cell, the zygote. In the sperm most histones are replaced by protamines and other nuclear basic proteins during spermiogenesis.<sup>27</sup> After the sperm entry into the oocyte cytoplasm, protamines are removed and replaced by nucleosomal histones. It has been shown that H3.3 accumulates preferentially in newly assembled chromatin on the paternal genome.<sup>25,27,28</sup> This constitutes the only event of genome wide deposition of H3.3 in the life of an organism. As a consequence of protamine exchange and the global acquisition of newly incorporated histones on the paternal genome, the paternal chromatin must acquire most epigenetic signatures, while the maternal genome has already epigenetic marks, which were acquired mainly during folliculogenesis.<sup>29</sup>

### Histone H3 Variants: Time-Window for a Transition

We can suggest different hypotheses concerning a potential role for H3.3 during reprogramming, perhaps through facilitating this process at the nucleosomal level. First, because in both PGCs and in the zygote reprogramming takes place—at least initially— independently of DNA replication, H3.3 deposition in PGCs and in the paternal chromatin might occur only because H3.1/2 is not available. This is however unlikely, as we have performed microinjections of H3.1 in zygotes before pronuclear formation and have not observed incorporation of H3.1 before the S-phase (our unpublished data). Thus, in the mouse zygote, as it is in *Drosophila*,<sup>30</sup> the non-incorporation of H3.1 might not only be due to the absence of the H3.1 protein, but rather to the absence of functional H3.1 deposition machinery outside of the S-phase. If we consider that H3.3 is deposited on the genome only because H3.1 cannot be deposited, then one could predict that H3.3 will play no particular role by itself but will just have a “neutral” or “default” filling-in role. Broadly speaking, this should be true if the deposition of H3 variants is genome-wide and not ‘loci-specifically’ regulated. However, we have recently found that in the mouse zygote, H3.3 and H3.1 show different distributions along specific genomic regions in the two pronuclei (Santenard A, et al. submitted). This new data suggests that H3.3 could have specific roles in the establishment of new epigenetic signatures in the male pronucleus after fertilization. If we consider that H3.3

can have both a “neutral” effect at some loci and specific effects on reprogramming of other loci, this would predict that H3.3 has to be recognised by different complexes at these different specific locations. If the genomic regions where specific variants become incorporated are important, the relevant question is how is this targeted deposition regulated? Is this achieved through the same HIRA complex? or Would there be a specific deposition complex, maybe different from the HIRA complex, in specialised genomic regions? And if so, would this putative complex recognise transcription factor sites or would it work based on RNA-interactions and/or bridging proteins? Of note, some histone chaperones can physically interact with specific transcription factors: HIRA has been shown to interact with Pax3 and Pax7, and NAP1 with E2F.<sup>31,32</sup> This suggests that deposition at given target genes can be potentially achieved through these interactions.

There is some evidence of H3.3 being enriched in specific genomic locations in developmentally regulated processes. During spermatogenesis, the X and Y chromosomes partially synapse to form the sex body in a process called Meiotic Sex Chromosome Inactivation (MSCI). During this process X and Y chromosomes become transcriptionally silenced.<sup>33</sup> In mouse and human, immediately after the initiation of MSCI at the early pachytene stage of meiotic prophase, nucleosomes present at the sex chromosomes are removed and replaced by new ones. Because this incorporation of new nucleosomes occurs independently of DNA synthesis, it is exclusively H3.3 that is incorporated in a chromosome-wide manner.<sup>34</sup> These observations have interesting consequences: firstly, that deposition of H3.3 can be specifically regulated and secondly, that H3.3 can also be associated with silenced, heterochromatic genomic regions or, at least, that this variant does not interfere with the heterochromatic state of the sex chromosomes. Whether H3.3 deposition is potentially involved in silencing is unclear. During MSCI, the incorporation of H3.3 and presumably newly assembled nucleosomes into chromatin further coincide with a lack of detection of almost all of the histone PTMs.<sup>34</sup> This also occurs during reprogramming of PGCs, where there is a loss of almost all PTMs between E11.5 and E12.5.<sup>26,35</sup> At this precise time, the replacement variant of H2A, H2A.Z, is also detected on the chromatin.<sup>26</sup> Thus, although it is currently impossible to show whether an individual nucleosome contains both H3.3 and H2A.Z variants, there is a time window at ~E11.5 during which H2A.Z and H3.3 are presumably both present in the nucleosomes. Of note, nucleosomes containing H2A.Z and H3.3 are less stable than those containing the canonical H2A and H3.1.<sup>24</sup> Because H2A.Z-H3.3-containing nucleosomes are less stable, the cell will thus need less energy to remove these. Thus, replacing the existing nucleosomes with their PTMs by less stable ones could be an efficient way to quickly remove old epigenetic marks and rapidly allow a huge reprogramming of the genome. Non-stable nucleosomes could also be a signal for the cell to ‘realise’ that something is ongoing at these loci and that some proteins have to come to replace these non-stable octamers by another ones that should thereafter remain as a long-term message. For reprogramming, H3.3 could then be a mark of transient state while H3.1/2 a long-term mark. Thus, another hypothesis concerning the role of H3.3 incorporation during reprogramming relates to the flexibility imparted by its potential ‘transient’ or ‘less stable’ state.

## Histone H2A Variants: Nucleosomal Partners for H3.3?

It is important to ask which are the nucleosomal partners of H3.3 during these developmental processes, in particular because it has been suggested that the identity of the partners within the nucleosome might give rise to different effects on the chromatin.<sup>24</sup> Variants of H2A are also involved in wide-scale chromatin remodelling events and have acquired specialised functions during development. Among these, phosphorylation of the H2A variant H2A.X was implicated in the initiation of the MSCI.<sup>36</sup> H2A.X is the most abundant H2A variant in *Xenopus* eggs, and the ability to remodel the sperm nucleus to form a paternal pronucleus after fertilization is directly associated with its phosphorylation status.<sup>37</sup> Moreover, unusually high levels of phosphorylated H2A.X are present throughout mouse preimplantation development (Ziegler-Birling et al., in press), opening the possibility for a role of this variant in chromatin assembly and remodelling during these early stages.<sup>58</sup> MacroH2A is an H2A variant possessing a long C-terminal domain and is conserved in vertebrates.<sup>38</sup> MacroH2A is enriched in the inactive X chromosome and becomes enriched in X and Y chromosomes during MSCI.<sup>39,40</sup> It is also peculiar because it shows a very tight temporal specific pattern of expression in mouse development. MacroH2A is present in the chromatin of developing and mature oocytes but it is immediately lost from the maternal chromatin in the zygote following fertilization and reappears only after the 8-cell stage.<sup>41</sup> Because macroH2A inhibits chromatin remodelling—and transcription<sup>42</sup>—it is possible that its rapid disappearance following fertilization is necessary to render the zygotic chromatin permissive for remodelling and epigenetic reprogramming.

We have mentioned above a third variant of H2A, H2A.Z, which is associated with both euchromatin and facultative heterochromatin.<sup>43</sup> H2A.Z has been implicated in apparently opposing functions such as prevention of gene silencing in *S. cerevisiae* and the establishment of heterochromatin in *Drosophila*.<sup>44,45</sup> Like H3.3, H2A.Z is not uniformly distributed throughout the genome. H2A.Z is enriched at promoters of developmentally regulated genes in chicken erythroid cells and in mouse ES cells.<sup>46,47</sup> Interestingly, H2A.Z localization is different in pluripotent versus differentiated cells.<sup>43</sup> In mouse ES cells, H2A.Z is required for differentiation and is functionally linked to Polycomb-induced gene silencing.<sup>47</sup> These findings are in full agreement with the early embryonic lethality resulting from loss of H2A.Z, as mouse blastocysts lacking H2A.Z fail to survive beyond implantation.<sup>48</sup> As we have already stated, a direct relationship between H2A.Z-H3.3-containing nucleosomes and local changes in chromatin structure have been documented. There are some interesting links between H3.3 and H2A.Z (H2Av in *Drosophila*). Nucleosome eviction during transcription can result in loss of H3.3/H2A.Z nucleosomes.<sup>49</sup> Given the low stability conferred by the presence of both H3.3 and H2A.Z to the nucleosome compared to H2A- or H3-containing nucleosomes, it has been suggested that H3.3 possesses a specific regulatory role when coupled with H2A.Z.<sup>24</sup> Another level of regulation by H2A variants arises from the interrelation of H2A.Z with DNA methylation. The presence of H2A.Z and DNA-methylation seem to be mutually exclusive in plants, and most importantly, loss of H2A.Z deposition following SWR1 complex knock-down leads to genomic hypermethylation. This led to the suggestion that H2A.Z protects genes from DNA

methylation.<sup>50</sup> Thus, it is clear that histone variants have an important role in establishing functional chromatin domains *in vivo*.

Because of all the above, we can suggest that a different complement of variants within the nucleosome core particle and the localised distribution of such variants are key elements to regulate epigenetic phenomena during development. Insight into the mechanisms governing histone variant deposition will therefore be central to our understanding of mammalian development. In particular the role for chaperones or deposition complexes with specificity towards H3.3 such as Chd1,<sup>51</sup> and of Chtz1/SWR1 for H2A.Z<sup>52</sup> in early development and whether there is a functional relation between the two types of deposition complexes.

## Can Histone Variants Leave a Mark on the Transmission of Epigenetic Information?

In human sperm, around 15% of the DNA remains wrapped around histones.<sup>53</sup> The same applies to the mouse sperm, albeit with a lower proportion (~4%). Concerning H3 variants, H3.1, H3.2 and H3.3 are all three present in human sperm.<sup>54</sup> Nothing is known about the specific differences of incorporation or the potential role that could be played by the variants in sperm. However, it has been shown that nucleosomal histones do not localise to random genomic regions.<sup>55</sup> It was demonstrated for the  $\beta$ -globin domain that loci bound by histones within this domain are preferentially those, which will be transcribed during early development and that silenced genes will be preferentially bound by protamines.<sup>56</sup> More recently, it was shown that sperm derived histones in human—at least H3.1 and H3.2—will contribute to the formation of nucleosomes in the paternal genome. If the observations of the  $\beta$ -globin locus can be generalised, we can think that histones are retained at genes that will be the first to be transcribed during zygotic genome activation. This is probably because the chromatin is less condensed with histones than with protamines, and as a consequence, the genes will be more easily, and more quickly transcribed after fertilization. Another role for these inherited histones could be to mark imprinted genes. After fertilization, when nearly the entire paternal genome is wrapped around H3.3-containing nucleosomes, remaining H3.1/2-containing nucleosomes could constitute a mark recognised by the cell. This has also been suggested in plants, where the paternal variant of H3 is carried by the sperm and will be maintained in the paternal genome in the endosperm for several cell divisions. The authors suggest that this phenomenon contributes to the parental genomic imprinting that occurs in the endosperm.<sup>57</sup> While all these hypotheses appear attractive, work is still to be done to test them directly in the mouse embryo.

## Conclusion

While deposition pathways and stability features of the different H3 variants are, up to now, assumed to be the same between somatic cells, germ cells and embryos, different roles for these variants in different cell types start to emerge. First, in somatic cells, H3.3 was described to be specifically linked with active transcription, while in germ cells and embryos, the replacement variant could be present as well in heterochromatin. Second, in somatic cells, H3.1/2 are known to be the most abundant variants, but in germ cells and embryos, because of a large scale replacement of nucleosomes that occurs

independently of replication, H3.3 will probably be more broadly present and will play an important role in reprogramming. The fact that H3.3 participates in the formation of less stable nucleosomes compared to H3.1 could be an advantage in germ cells and embryos, not only to allow transcription as it is in somatic cells, but also to allow quick and efficient removal of existing epigenetic marks, acquisition of new ones and a more open and permissive chromatin conformation. Thus, H3.1/2 would rather play a role in the propagation of a stable epigenetic state, a mark for example, through mitosis. We can also suggest that, in an H3.3 background in the paternal genome in zygotes, the presence of H3.1/2 could act as ‘marks’ by themselves, as H3.3 does in specific (e.g., transcribed) loci in a broad H3.1/2 background in somatic cells. If we assume that the same protein has different roles in a same organism, this implies that there should be different complexes that will interact with each of the variants depending on its localization or on its interacting proteins. Up to now, only one complex containing the histone chaperone HIRA has been described to interact with and deposit H3.3 but further investigations are needed to understand the pathways through which H3.3 could regulate different chromatin conformations at different loci. Thus, we suggest that H3.3 has an important role in setting up a ‘transition’ signature in germ cells and embryos, and provides the possibility to infer changes on the chromatin independent of one of the most basic DNA process, DNA replication. This should confer flexibility during important developmental processes to support changes mediated via alterations in chromatin composition.

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