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Histone variant selectivity at the transcription start site

H2A.Z or H2A.Lap1

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Considerable attention has been given to the understanding of how nucleosomes are altered or removed from the transcription start site of RNA polymerase II genes to enable transcription to proceed. This has led to the view that for transcriptional activation to occur, the transcription start site (TSS) must become depleted of nucleosomes. However, we have shown that this is not the case with different unstable histone H2A variant-containing nucleosomes occupying the TSS under different physiological settings. For example, during mouse spermatogenesis we found that the mouse homolog of human H2A.Bbd, H2A.Lap1, is targeted to the TSS of active genes expressed during specific stages of spermatogenesis. On the other hand, we observed in trophoblast stem cells, a H2A.Z-containing nucleosome occupying the TSS of genes active in the G_1 phase of the cell cycle. Notably, this H2A.Z-containing nucleosome was different compared with other promoter specific H2A.Z nucleosomes by being heterotypic rather than being homotypic. In other words, it did not contain the expected two copies of H2A.Z per nucleosome but only one (i.e., H2A.Z/H2A rather than H2A.Z/H2A.Z). Given these observations, we wondered whether the histone variant composition of a nucleosome at an active TSS could in fact vary in the same cell type. To investigate this possibility, we performed H2A.Z ChIP-H2A reChIP assays in the mouse testis and compared this data with our testis H2A.Lap1 ChIP-seq data. Indeed, we find that different promoters involved in the expression of genes involved in distinct biological processes can contain either H2A.Z/H2A or H2A.Lap1. This argues that specific mechanisms exist, which can determine whether H2A.Z or H2A.Lap1 is targeted to the TSS of an active gene.

Keywords: active chromatin, nucleosome positioning, the transcriptional start site, histone variants, H2A.Z, H2A.Lap1, H2A.Bbd, spermatogenesis, stem cells, ChIP-seq, microarray gene expression analysis

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Introduction

Eukaryotic genomic DNA is assembled into a highly compacted state with an equal mass of protein to form chromatin. The repeating unit of chromatin is the nucleosome, which is formed by wrapping ~150 base pairs of DNA around a core histone octamer (two molecules each of histone H2A, H2B, H3, and H4). Nucleosomes are connected by short linker DNA segments to form long arrays, which undergo short-range intra and long-range inter-nucleosomal interactions to contribute to the high degree of compaction observed in interphase chromosomes.[1] This condensation of genomic DNA into chromatin has profound implications for the regulation of key DNA processes such as transcription. For example, this compaction and occupancy of canonical nucleosomes at the TSS of genes transcribed by RNA polymerase II prevents access to the transcription machinery.[2-4] It therefore has been assumed that for transcription to proceed, a nucleosome free (NFR) or depleted region (NDR) must be created at the TSS,[5-7] and the experimentally observed increase in accessibility to micrococcal nuclease digestion has been

used as evidence to support this notion.[7,8] However, such sensitivity to micrococcal nuclease digestion at the TSS does not necessarily imply a loss of nucleosomes but could also be the consequence of the formation of a highly unstable nucleosome.[7] Indeed, our work[9,10] and the studies of others[11] indicate that this is the case and that histone variants play an important role in assembling unstable nucleosomes at the TSS.

H2A.Z/H2A.Z Homotypic vs. H2A.Z/H2A Heterotypic Nucleosomes

Among the core histones, the H2A family shows the greatest divergence in primary sequence leading to the greatest number of variants known.[12] The key amino acid differences between histone H2A variants and histone H2A are strategically placed within the nucleosome to affect nucleosome stability[13-15] and also on the surface of the nucleosome (the acidic patch) to regulate the interactions between neighboring nucleosomes thereby determining the extent chromatin compaction.[1,10,12,16,17] A highly conserved and extensively studied histone variant is H2A.Z.[12] H2A.Z has a range of functions including being required for early metazoan development,[18-20] chromosome organization and inheritance,[21-23] as well as regulating promoter chromatin architecture.[24,25] Notably in mammalian cells, the apparent NDR of active genes at the TSS is flanked on both sides by H2A.Z containing-nucleosomes (the -2 and +1 nucleosome, respectively).[24,25]

A long-held view has been that chromatin-based information at the promoter, including H2A.Z, are stable marks that provide "transcriptional memory" so that following cell division, a previously active Pol II promoter can be readily re-activated.[26] We tested this hypothesis by examining how the two H2A.Z-containing nucleosomes that flank the TSS (in particular the H2A.Z nucleosome located just downstream of the TSS, which is referred to as the +1 nucleosome) are restored following the highly disruptive process of DNA replication (S phase) and whether they remain with chromosomes as they condense during metaphase (M phase).[9] We employed mouse trophoblast stem (TS) cells as a model system, given that H2A.Z is required for their viability.[19]

Experimentally, we synchronized cells at the G_1, S and M phases of the cell cycle and then performed H2A.Z and histone H3 ChIP-seq experiments as well as examining global gene expression at these three stages of the cell cycle using whole mouse genome expression microarrays. Unexpectedly, compared with histone H3, there was a loss of H2A.Z at active promoters in S and M vs. G_1 for all groups of genes irrespective of whether they were more highly expressed at G_1, S or M phase.[9] The next question therefore became why was H2A.Z not fully restored at promoters following S phase to G_1 levels?

To address this problem we then investigated how H2A.Z-containing nucleosomes were inherited following S phase by determining whether the number of copies of H2A.Z per nucleosome changed (a H2A.Z nucleosome could contain either one or two copies of H2A.Z). Specifically, we investigated whether at the promoter there was an increase in heterotypic H2A.Z-H2A nucleosomes or would the ratio of heterotypic H2A.Z-H2A to homotypic H2A.Z-H2A.Z remain unchanged following S phase.

To investigate this issue we performed H2A.Z ChIP followed by H2A reChIP assays.[9] Basically, ChIP assays were first performed using affinity purified H2A.Z antibodies to immunoprecipitate all H2A.Z-containing nucleosomes followed by a second ChIP employing H2A antibodies to pull down heterotypic H2A.Z-H2A nucleosomes. Significantly, we observed that only ~5% of H2A.Z nucleosomes at G_1 were heterotypic but by M phase, this dramatically increased to ~35%. At active promoters, there was a clear increase in heterotypic H2A.Z-H2A nucleosomes at S and M compared with G_1 phase (compare **Fig. 1B and C**, respectively with **Fig. 1A and D**). This allowed us to conclude that at G_1, homotypic H2A.Z-H2A.Z nucleosomes flanked both sides of the TSS but following the disruptive DNA replication process, these nucleosomes became heterotypic, which can explain, at least in part, why H2A.Z was not fully restored at promoters to G_1 levels following S phase.

Surprisingly, we found that heterotypic H2A.Z-H2A nucleosomes do also exist at G_1 and most notably these nucleosomes were located on the TSS of active promoters[9] (**Fig. 1A**). This demonstration that the TSS is not nucleosome free in TS cells is supported by a previous finding showing that a double histone variant nucleosome comprised of H2A.Z and H3.3 was located at the TSS of active genes in HeLa cells.[11] Moreover, while it was revealed that this double variant nucleosome was unstable,[27] the structural basis for its instability remained unknown.[28] Our demonstration that this unstable nucleosome located at the TSS is heterotypic in respect with H2A.Z may offer a molecular explanation for its instability.

Based on the crystal structure of a homotypic H2A.Z–H2A.Z nucleosome, it was predicted that the replacement of H2A with only one molecule of H2A.Z will cause a major structural clash between their L1 loop regions, which is sufficient to destabilize it.[28,29] Consistent with this heterotypic nucleosome being labile, we also found it at other dynamic genomic loci including CTCF binding sites and DNase I hypersensitive sites. In addition, we observed displacement of this heterotypic H2A.Z/H2A nucleosome following the passage through S phase at all of these sites.[9] On the other hand, it is worth noting that a homotypic H2A.Z/H2A.Z nucleosome is more stable than a canonical nucleosome, which can be attributed to an enhanced interaction between the L1 loop regions of each H2A.Z molecule compared with H2A.[15] Why a heterotypic H2A.Z/H2A nucleosome only exists at the TSS in G_1 is unclear but probably reflects a different chromatin configuration at the TSS for genes expressed in G_1 vs. genes expressed in M and S.[9]

Finally, we addressed the question of why heterotypic H2A.Z-H2A nucleosomes formed during S phase was not fully restored to the homotypic state by M phase (**Fig. 1B and C**, respectively; **Fig. 1D**). Another important function of H2A.Z is to maintain the structural integrity of the centromere and surrounding constitutive

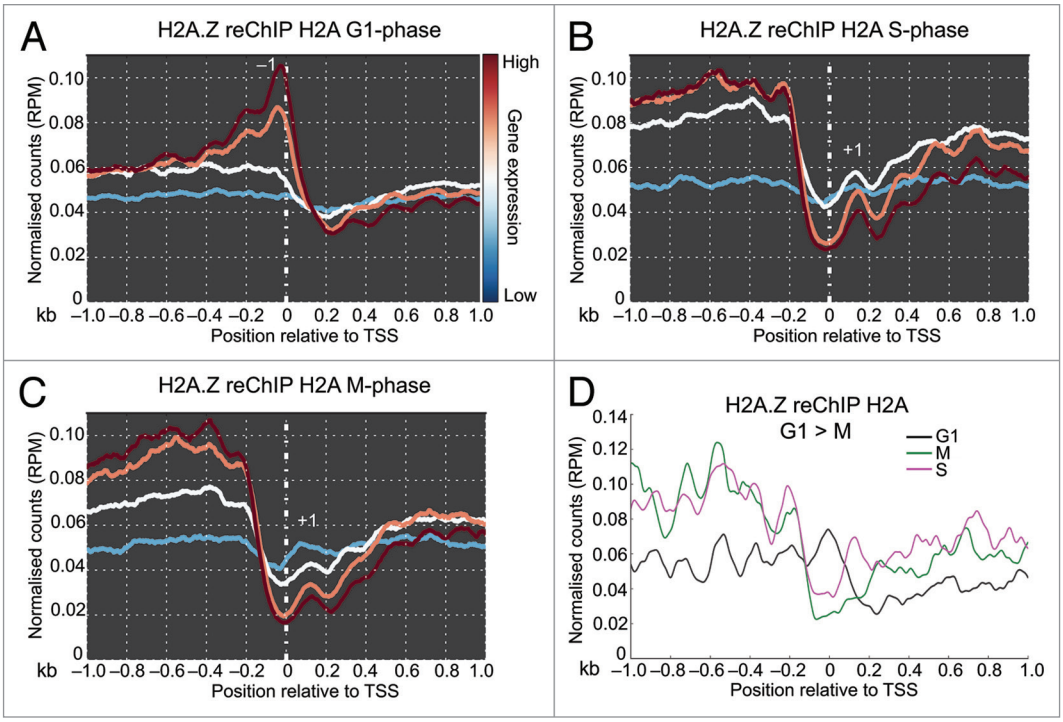

Figure 1. (**A**) Four H2A.Z ChIP-H2A reChIP profiles were generated for the G_1 phase. Each individual line represents a group of 4200 genes and represents the normalized tag counts at each base pair, aligned between -1 and +1 kb from the TSS. The line color reflects the average gene expression rank of the contained genes as shown. (**B**) H2A.Z ChIP-H2A reChIP normalized tag counts at S phase for 4 groups of 4200 genes aligned with the TSS. (**C**) H2A.Z normalized tag counts at M phase for 4 groups of 4200 genes aligned with the TSS. (**D**) We chose the top 100 differentially expressed genes for genes more highly expressed in G_1 phase vs. M phase and represented them as a single line of normalized H2A.Z ChIP-H2A reChIP counts at each base pair, aligned with the TSS. How this profile changes as the TS cells progresses from G_1 to M is shown. What is clearly observed is that H2A.Z-H2A heterotypic nucleosomes form at the promoter at S phase and that this profile remains similar at M phase.

heterochromatin.[22,23] We therefore wondered whether the loss of H2A.Z at promoters was coincident with a gain of H2A.Z at centromeres and indeed this was found to be the case. Taken together, this dynamic net transfer of H2A.Z from promoters to the centromere at M phase brings into question whether H2A.Z has a role in providing transcriptional memory during the cell cycle. Interestingly, it has also been shown that histone post-translational modifications and other histone variants are likewise restored slowly after DNA replication, and in some cases requiring more than one round of cell division to be fully re-established.[30-32]

H2A.Lap1 and Spermatogenesis

While H2A.Z is ubiquitously expressed, human H2A.Bbd (H2A.B)[33] is expressed primarily in the adult testis and brain (NCBI GEO data sets). It is also aberrantly expressed in a number of tumors and cancer cell lines[34] (NCBI GEO data sets). In contrast to H2A.Z which promotes nucleosome stability[15] and chromatin compaction,[1] H2A.Bbd-containing histone octamers and mononucleosomes are unstable and the latter only protects ~120 base pairs of DNA from micrococcal digestion compared with 145 base pairs for canonical nucleosome core particles.[13,35] This is consistent with FRET, AFM, and cryo-EM studies showing the unwrapping of nucleosomal DNA from the nucleosome surface at the DNA entry and exit points.[13,35,36] Assembly of nucleosomal arrays with H2A.Bbd completely inhibits array folding and compaction leading to a de-repression of chromatin mediated transcription.[17] This ability of H2A.Bbd to prevent chromatin compaction is due to the loss of three acidic amino acid residues, which partially neutralizes the acidic patch located on the surface of the nucleosome.[17]

To gain new insights into the possible role(s) of H2A.Bbd and its orthologs, we turned to the mouse where we identified four major "H2A.Bbd-like" proteins. We designated these variants as H2A.Lap 1–4 (**l**ack of an **a**cidic **p**atch)[10] (H2A.B.1, H2A.L.1, H2A.L.2, and H2A.M, respectively)[33] to more accurately reflect their partially neutralized acidic patch, with H2A.Lap1 being most similar to H2A.Bbd. Analogous to H2A.Bbd, H2A.Lap1 is expressed primarily in the testis and the brain.[10] Intriguingly though, while H2A.Lap1 also prevented chromatin compaction and also only protected ~120 base pairs of DNA from micrococcal digestion in vitro, it was less efficient in decompacting chromatin compared with H2A.Bbd indicating that these histone variants are not completely functionally equivalent (which may explain why the mouse has four major "H2A.Bbd-like" proteins whereas human has only one protein).

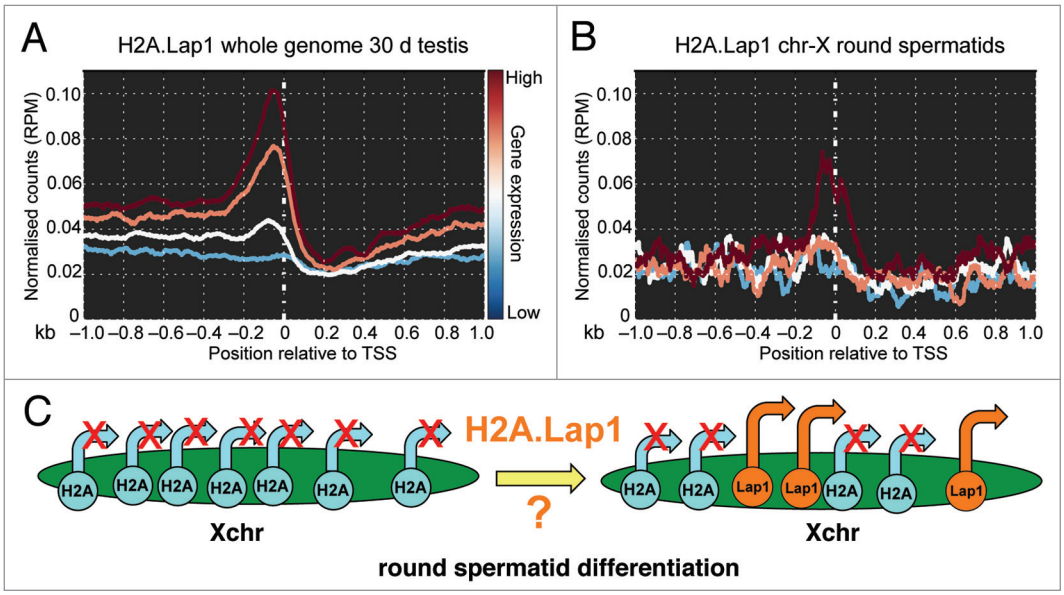

Figure 2. (**A**) 4 H2A.Lap1 ChIP profiles were generated for the 30 d testis. Each individual line represents a group of 4,700 genes representing the normalized tag counts at each base pair, aligned between -1 and +1 kb from the TSS. The line color reflects the average gene expression rank of the contained genes. (**B**) Using published round spermatid X chromosome expression data,[37] four individual H2A.Lap1 ChIP profiles were generated. Each individual line represents a group of 140 genes. (**C**) A model depicting the specific targeting of H2A.Lap1 to the TSS of the small number of genes on the X chromosome that become specifically activated during the latter stages of round spermatid differentiation. We propose that the replacement of H2A with H2A.Lap1 destabilizes chromatin at the TSS to facilitate the recruitment of the Pol II machinery. Unknown (?) is the H2A.Lap1 loading complex that targets H2A.Lap1 to the TSS.

Table 1. Summary of GO Biological Process enrichment and Functional Clustering for the top 250 genes enriched with either H2A.Lap1 or H2A.Z-H2A at their TSS

H2A.Lap1	H2A.Z–H2A heterotypic
Gene Ontology Enrichment by Biological Process and Functional Clustering	
Gene Expression	**Metabolic processes**
Transcription, DNA-dependent	DNA strand renaturation*
Translation	Annealing helicase activity*
RNA metabolic process	TOR signaling pathway
RNA biosynthetic process	Mitochondrial ribosomal protein (F)
Spermatogenesis	Cytoskeleton organization(F)
Transcriptional mediation and TATA-box binding (F)	Mitotic cell division (F)

Processes were observed with GOrilla[44] and Gprofiler.[45] Parent processes are in bold. Processes marked with * were not significant after correction for False Discovery Rate.[46] Items marked with (F) were major components of functional clusters observed by DAVID.[47]

Mammalian spermatogenesis is a complex developmental and differentiation process that occurs in seminiferous tubules. A rapidly dividing pool of stem cell spermatogonia is located at the outer edge of these tubules. Some spermatogonia stop dividing and differentiate into primary spermatocytes, which enter the first meiotic prophase. The first meiotic prophase consists of five sequential stages: leptotene (condensation of interphase chromosomes), zygotene (synapsis begins), pachytene (recombination and crossing over occurs), diplotene (desynapsis initiates), and diakinesis (transition to metaphase I). Cells then complete division I of meiosis to produce secondary spermatocytes, which then undergo meiotic division II to produce haploid round spermatids. Differentiation (spermiogenesis) continues toward the center of the tubule where mature spermatozoa ultimately escape into the lumen.

To determine precisely which mouse testis cell types express H2A.Lap1, we performed indirect immunofluorescence analyses using affinity purified H2A.Lap1 antibodies on testis sections, displaying all 12 stages of the cell cycle of the seminiferous tubule, as well as on surface spreads of individual spermatocytes and spermatids. We found that H2A.Lap1 is not ubiquitously expressed throughout spermatogenesis but is expressed in a temporally specific manner, between pachytene and the round spermatid stage of spermatogenesis.[10]

To investigate the genomic location of H2A.Lap1 and its link with transcription, we performed H2A.Lap1 ChIP-seq experiments and examined global gene expression using whole mouse expression microarrays in the 30 d testis (where the vast majority of tubules contained round spermatids). The striking observation was that H2A.Lap1 was located at the TSS of active genes analogous to the position of heterotypic H2A.Z/H2A nucleosomes in TS cells (**Fig. 2A**). This allowed us to conclude that H2A.Lap1 is a new component of genes expressed during spermatogenesis and moreover, confirmed

the view that the TSS of an active gene is not nucleosome free. Taken together, our results show that H2A.Z or a H2A.Lap1-containing nucleosome can be precisely positioned at the TSS dependent upon their physiological context.

Our immunofluorescence analysis of round spermatids revealed an unexpected observation; while H2A.Lap1 was largely excluded from heterochromatin regions, as we expected, it was targeted to the inactive X or Y chromosome specifically during the latter stages of round spermatid differentiation.[10] In mammals, the X or Y chromosome are subjected to meiotic sex chromosome inactivation during pachytene and significantly, this silencing of the sex chromosomes persists postmeiotically throughout spermatogenesis.[21,37,38] However, Namekawa and colleagues showed that a small number of genes on the X chromosome, required for spermatogenesis, are reactivated in round spermatids (~13%).[37]

Based on this knowledge, our hypothesis therefore became that H2A.Lap1 was specifically targeted to these X-linked and round spermatid specific genes to enable their activation. To test this hypothesis, we used our H2A.Lap1 ChIP-seq data and combined it with published X chromosome gene expression data in round spermatids.[37] Confirming our hypothesis, we found that H2A.Lap1 was not present on inactive genes but selectively enriched at the TSS of X chromosome genes that were only active in round spermatids (**Fig. 2B**). Moreover, maximal transcription was coincident with the highest H2A.Lap1 signal at their transcription start site at the latter stages of round spermatid differentiation suggesting that H2A.Lap1 is directly involved in the transcriptional activation process.[10] Combined with our structural data, this allowed us to propose a model whereby H2A.Lap1 coordinately activates transcription of genes on the X chromosome by creating an open chromatin configuration at the TSS (**Fig. 2C**). A major problem for the future is to uncover the specific H2A.Lap1 chaperone and/or ATP-dependent remodelling complex that targets H2A.Lap1 to the TSS (see below).

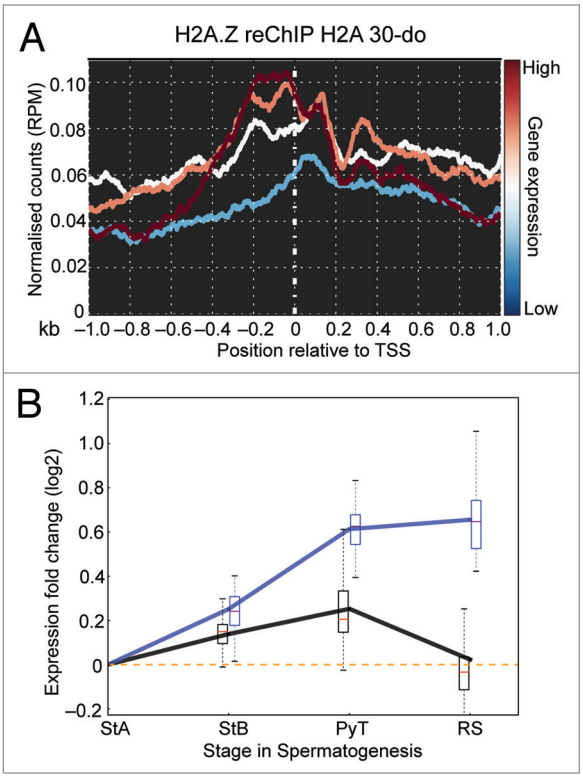

Figure 3. (**A**) Four H2A.Z ChIP-H2A reChIP profiles were generated for the 30 d testis. Each individual line represents a group of 3200 genes and represents the normalized tag counts at each base pair, aligned between -1 and +1 kb from the TSS. The line color reflects the average gene expression rank of the contained genes as shown. (**B**) Log2 change in gene expression during selected stages of spermatogenesis (StA, Spermatogonium type A; StB, Spermatogonium type B; PyT, Pachytene; RS, Round Spermatid) for the top 250 genes containing the strongest enrichment signals for either H2A.Lap1 or H2A.Z-H2A at their TSS. Blue lines and boxes are enriched for H2A.Lap1; black lines and boxes are enriched for heterotypic H2A.Z-H2A nucleosomes. Solid lines show the mean change in expression normalized to the StA stage. Box plots show the upper and lower quartiles, and the median expression at each stage. The box's whiskers span the 95% confidence interval. To perform this analysis, we used published gene expression data from Namekawa and colleagues[37] as described previously.[10]

A Promoter Specific Choice Between H2A.Z and H2A.Lap1

Given our observation that heterotypic H2A.Z-H2A nucleosomes exist at an active TSS in TS cells (**Fig. 1A**), we wondered whether such heterotypic nucleosomes might also be present on promoters activated during spermatogenesis and if so, whether they are on active promoters during the early stages of spermatogenesis before being replaced by H2A.Lap1 at pachytene. Alternatively, perhaps both types of histone variant-containing nucleosomes can be present at same time during and following the pachytene stage but on different genes involved in distinct biological processes. Both possibilities are not mutually exclusive, however.

To answer the first question we performed H2A.Z ChIP H2A reChIP assays using 30 d testes and combined this data with our gene expression analysis. All mouse genes were separated into quartiles according to their expression level. A clear positive correlation between the abundance of a heterotypic H2A.Z-H2A nucleosome at the TSS and the level of expression is clearly observed analogous to active promoters in TS cells (**Fig. 3A**). Interestingly, the heterotypic H2A.Z-H2A nucleosome is not uniquely positioned at the TSS but is present at several locations around the TSS including at nucleosome position –2 and +1 indicating a different chromatin structure for genes active in the mouse testis compared with TS cells. Indeed, we previously performed H2A.Z

ChIP experiments in the 30 d testis and reported the unexpected observation that while H2A.Z was broadly enriched at the -2 nucleosome position, it was depleted at the +1 position.[10] This suggests that in addition to the TSS, the +1 nucleosome is also unstable being predominantly in the heterotypic form. We conclude that a heterotypic H2A.Z-H2A nucleosome is present at the TSS in the testis arguing that this special heterotypic nucleosome may be a universal feature of an active mammalian gene.

To address the second possibility that an active promoter can selectively choose between H2A.Lap1 or H2A.Z, we took the top 250 genes in the 30 d testis enriched with either a H2A.Lap1-containing nucleosome or a heterotypic H2A.Z-H2A-containing nucleosome at their TSS, and examined the relative level of expression of these 250 genes at four stages of spermatogenesis (spermatogonia stage A, spermatogonia stage B, pachytene, and the round spermatid stage) normalized to the level of expression at spermatogonia stage A using published gene expression data[37] (**Fig. 3B**). Significantly, these top 250 heterotypic H2A.Z-H2A or H2A.Lap1-nucleosome containing genes were different (**Table 1**).

We find that heterotypic nucleosomes are present on genes active at different stages of spermatogenesis with the normalized mean expression for the top 250 heterotypic H2A.Z-H2A-containing genes peaking at the pachytene stage (**Fig. 3B**). The normalized mean expression of H2A.Lap1-containing genes also increases at pachytene but this increase is more significant compared with heterotypic containing genes. In addition, this level of expression is maintained in the round spermatid stage, which is expected given that H2A.Lap1 is expressed between pachytene and the round spermatid stage (**Fig. 3B**). It is worth highlighting that the presence of H2A.Lap1 at the TSS of genes active in the round spermatid stage is not due to a simple mass action effect because the expression of H2A.Z protein actually peaks in round spermatids.[21]

Given that the genes that contained either H2A.Lap1 or H2A.Z-H2A were different, we wondered whether their respective genes were involved in different biological processes. To investigate this possibility, we performed gene ontology analysis on these two groups of genes. While H2A.Z-H2A heterotypic nucleosomes were associated with genes that display a range of different functions, such as metabolic processes, no single process displayed a significant enrichment of H2A.Z-H2A indicating that this histone variant nucleosome has a more ubiquitous distribution not involved in any specific biological process (**Table 1**). On the other hand, processes involving transcription, RNA metabolism, and spermatogenesis were significantly overrepresented among H2A.Lap1 enriched genes (**Table 1**).

Taken together, these new observations show that during the same stages of spermatogenesis the TSS of active genes can selectively contain either a H2A.Z-H2A heterotypic or a H2A.Lap1-containing nucleosome. This argues that specific mechanisms exist that can determine whether H2A.Z or H2A.Lap1 is targeted to the TSS. These mechanisms most likely involve histone variant specific chaperones and/or ATP-dependent remodelling complexes and promoter specific transcription factors. Experiments are in progress to identify the nature of such putative complexes specific for H2A.Lap1. Concerning H2A.Z, our hypothesis is that ATP-dependent complexes that load H2A.Z into chromatin (e.g., P400 or SRCAP[39,40]) will exchange only a single copy of H2A with H2A.Z when a nucleosome is positioned at the TSS (perhaps regulated by H3.3 incorporation[28] and/or histone acetylation[41]). Alternatively, or in addition, ATP-dependent complexes that are capably of removing H2A.Z (e.g., INO80[42]) may function to exchange H2A.Z with H2A at the TSS to create an unstable heterotypic nucleosome.

The reason why the nucleosome composition at the TSS is regulated in this manner is unclear but the specific targeting of H2A.Lap1 may ensure genes are activated in the correct temporal and spatial manner and/or might facilitate higher levels of transcription compared with a H2A.Z-H2A heterotypic nucleosome. Indeed, the highest level of overall transcription does occur between pachytene and the round spermatid stage, which is when H2A.Lap1 is expressed.[43] Moreover, it is particularly intriguing that H2A.Lap1 is associated with genes that are, themselves, required for gene expression. In conclusion, we believe that this selectivity of histone variants at the TSS adds another layer of complexity to the process of transcriptional regulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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