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ATX1/AtCOMPASS and the H3K4me3 Marks: How Do They Activate *Arabidopsis* Genes?

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Abstract
Despite the proven correlation between gene transcriptional activity and the levels of tri-methyl marks on histone 3 lysine4 (H3K4me3) of their nucleosomes, whether H3K4me3 contributes to, or "registers," activated transcription is still controversial. Other questions of broad relevance are whether histone-modifying proteins are involved in the recruitment of Pol II and the general transcription machinery and whether they have roles other than their enzyme activities. We address these questions as well as the roles of the *ARABIDOPSIS* HOMOLOG OF TRITHORAX1 (ATX1), of the COMPASS-related (AtCOMPASS) protein complex, and of their product, H3K4me3, at ATX1-dependent genes. We suggest that the ambiguity about the role of H3K4me3 as an activating mark is because of the unknown duality of the ATX1/AtCOMPASS to facilitate PIC assembly and to generate H3K4me3, which is essential for activating transcriptional elongation.

Background: the Trithorax proteins as epigenetic regulators and chromatin modifiers

The Trithorax protein (Trx) has been recognized as an activator of the homeotic genes in *Drosophila* and as a component of the Trithorax group (TrxG) of developmental epigenetic regulators [1]. Trx-related proteins (annotated as KMT class enzymes carrying specific histone H3 lysine 4 methylating activities) were found broadly distributed in eukaryotes encompassing unicellular and multicellular domains of life [2]. Trx-homologs regulate diverse biological functions beyond development. Thus, in addition to maintaining the expression of *Hox* genes, the mammalian (the MLL) proteins are involved in differentiation,

in the regulation of the cell cycle, in senescence, in various diseases, and in cancer progression [3**] The plant homolog *ARABIDOPSIS* HOMOLOG OF TRITHORAX1 (ATX1) is also involved in pleiotropic functions including development, organogenesis [4,5] and responses to biotic [6] and abiotic [7,8] stresses.

TrxG and Polycomb (PcG) complexes are implicated in maintaining the transcriptional states of developmental genes throughout ontogenesis and are considered general carriers of cell memory [3**]. The molecular mechanisms of these functions, however, are still obscure. The merging of two fields, epigenetics and chromatin biochemistry, has made it clear that "the effects on gene expression observed in epigenetic phenomena and during development could only be fully understood in light of chromatin-based mechanisms" [9]. Chromatin is considered the main regulator of access to the underlying DNA sequences, and because histone modifications may affect chromatin structure and nucleosome placement, they are expected to influence the potential of DNA to serve as a template for transcription [10**,11].

Histone H3 lysine4 tri-methylation (H3K4me3) conferred by the Trithorax proteins and its homologs in different eukaryotic systems has been linked to the transcriptional activation of regulated genes suggesting that the molecular basis of the Trithorax effects is in its ability to modify histones and, thus, chromatin structure [12]. How histone modifications influence the transcriptional process, however, is still controversial, the central issue being causality [13**]. These authors raised the question of whether histone modifications are responsible for differences in transcriptional states, or whether differences in histone modifications occurring at the genes result from the dynamic processes (transcription or nucleosome remodeling) taking place and, thus, are a consequence, rather than, inducers of the processes. Whether H3K4me3 regulates, or simply "registers," active transcription is still ambiguous. The truly challenging question is to understand the mechanisms regulating transcription at the molecular level.

Here, we discuss the roles of H3K4me3 marks and of the ATX1/AtCOMPASS complex (which is essential for the H3K4me3 modification [14]) in the transcription of ATX1-dependent genes in *Arabidopsis*. The three key findings discussed are: (1) ATX1/AtCOMPASS have roles as co-activators at the promoters linked to the basal transcriptional machinery; (2) these roles do not depend on their enzyme activity; and (3) H3K4me3 is not required for TBP/Pol II recruitment to promoters but is a critical activating mark for transcription elongation. These findings are discussed in comparison with data available from yeast and animal systems. We suggest that the ambiguity about the role of H3K4me3 as an activating mark is due to the duality of the ATX1/AtCOMPASS functions to both facilitate PIC assembly and to generate H3K4me3 as an activating mark for the elongation [15**].

Distribution of H3K4me3, ATX1, AtCOMPASS, and Pol II at transcriptionally active genes

High levels of H3K4me3 form a peak at the 5′-regions of active genes and generally show a positive correlation with transcription rates and with the occupancy of activated RNA polymerase II phosphorylated at Ser5 [16*,17*,18*].

The causative link between H3K4me3 accumulation and transcriptional activation, however, has been particularly difficult to establish using genetically deficient mutants, as most of the KMTs (TRX-related proteins, in particular) are multidomain proteins (fig. 1) that may have roles beyond their enzyme activity. For example, the N-terminal cleavage product of the *Drosophila* Trx has been found distributed over large domains of active PcG-target genes lacking H3K4me3, while the C-terminal region containing the SET domain was found at sites that lacked H3K4me3 suggesting roles for the *Drosophila* Trx independent of the KMT activity [3**]. ATX1 carries multiple architectural domains (fig. 1) that play diverse functions as well. Thus, the ATX1-ePHD domain affects expression from specific ATX1-dependent genes through its ability to bind the lipid Phosphatidyl Inositol-5 Phosphate (PtdIns5P) influencing the nuclei-cytoplasm distribution of ATX1 [19,18*,20,21*]. Of note, this function of ePHD is different from the ability of PHD peptides (belonging in a distinct phylogenetic clade), which can bind H3K4me3 [22,23]. Furthermore, an ATX1 isoform containing only the SET domain (soloSET), with an exclusively cytoplasmic function, is encoded from the ATX1 locus illustrating products with different functions that are encoded by a KMT gene [24].

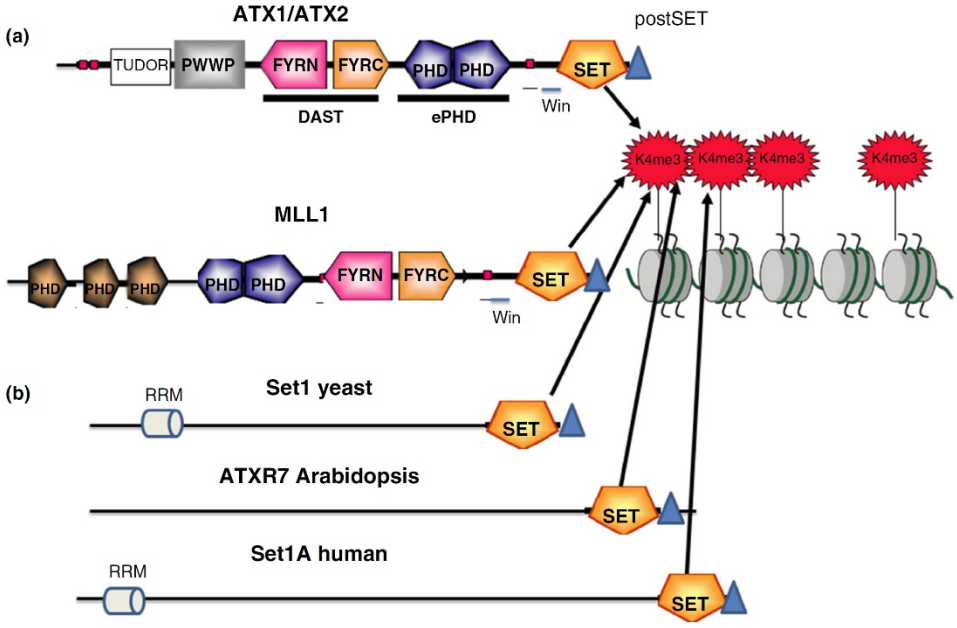

Figure 1. Molecular architecture of H3K4-methyltransferases (KMT). (a) Representative members of the TRX subfamily. (b) Representative members of the SET subfamily. SET domains from both subfamilies are involved in tri-methylation of Lys4 of Histone H3. The small red square upstream of the ATX1/ATX2 and MLL1 SET domains indicate the nuclear localization signal and the small line indicate the presence and location of the WDR-binding region, the Win domain.

Despite the potentially diverse roles played by the multidomain methyltransferases, a standard experimental approach is to delete/disrupt/knockdown the genes encoding the

KMTs or a subunit of the COMPASS/COMPASS-LIKE complexes that are critical for their activity. This approach causes depletion (full or partial) of a multidomain protein or complex, potentially disrupting more than one function. As a consequence, overlapping effects could obscure causative links or result in misleading interpretations. Using mutants with amino acid substitutions that specifically inactivate the enzyme functions of AXT1, while preserving its potential role in other functions, avoided this caveat and has allowed us to untangle the effects of a lack of H3K4me3 from those caused by the disruption of the structural integrity of the ATX1-AtCOMPASS complex [15**].

ATX1 forms a complex with AtCOMPASS at promoters in a function that does not require H3K4 methyltransferase activity

Wild type–level assembly of the pre-initiation complex (PIC) at the promoters of ATX1-regulated genes requires presence of ATX1 and AtCOMPASS. The conclusion is based on observations that in plants where the ATX1/AtCOMPASS presence at promoters is diminished (in *atx1* mutants or in RNAi AtCOMPASS subunit knockdown lines), the TATA-binding protein (TBP) and RNA Polymerase II (Pol II) accumulate at lower levels than in wild type [15**,18*]. In contrast, when an ATX1-set protein that lacks the enzyme activity but preserves the integrity of the ATX1/AtCOMPASS, the TATA-binding protein (TBP) and Pol II accumulate on promoters at levels similar to the wild type, despite greatly diminished H3K4me3 levels at the ATX1-regulated genes [15**]. These results demonstrated that a high-level presence of H3K4me3 was not critical for PIC formation or for promoter accessibility of ATX1 regulated genes.

Of note, MLL1 affected also PIC formation and Pol II occupancy at target genes in mammalian cells but the effects are apparently indirect as MLL1 establishes the H3K4me3 marks, which are subsequently bound by the PHD domain of the TAF3 subunit of TFIID [25**]. *Arabidopsis*, which lacks a gene for a putative TAF3 subunit underscores further the difference between the ATX1 and MLL models for their roles at the promoters of regulated genes [18*].

Most importantly, the results with ATX1-set mutants indicate ATX1/AtCOMPASS complex plays a role in PIC assembly that is distinct from its role in H3K4me3 formation. ATX1 accumulates at a promoter in genetic backgrounds depleted of AtCOMPASS subunits, suggesting interactions with other, proteins, for example PIC components, take place at the promoter [15**]. The finding of ATX1 in a complex with TBP/Pol II and the ability of ATX1 to bind directly the non-phosphorylated CTD of Pol II support this conclusion [18*].

Therefore, both ATX1 and AtCOMPASS are linked with the basal transcriptional machinery at promoters in a role that does not require H3K4 methyltransferase activity. These results illustrate novel aspects of the roles of ATX1/AtCOMPASS as transcriptional co-activators required for the PIC formation and/or stability during the initiation of transcription. The significance of this result is considered in the context of a broader current question of whether histone-modifying activities are required for the recruitment of Pol II and the general transcription machinery [26**]. In yeast, histone acetyl-transferases are required as transcriptional co-activators for optimal recruitment of the TATA binding protein and RNA polymerase II [27].

ATX1 migrates from the promoter to the +300 nt region to modify chromatin

After PIC formation and after becoming phosphorylated at Ser5P of its CTD, Pol II clears the promoter and shifts to the transcription start site (TSS), where it accumulates as a peak at about +300 nt. This step marks the transition from initiation to the elongation phase of transcription [28**]. Importantly, it is at this step that the activated (Ser5P) Pol II recruits ATX1 to this position forming its occupancy peak at ~+300 nt downstream of the TSS [18*]. This recruitment requires Ser5P Pol II as drug inhibition of the kinase responsible for Ser5 phosphorylation prevents ATX1 recruitment to the +300 nt position, without diminishing ATX1 recruitment to the promoter. Presumably, the recruitment of ATX1 to the +300 nt position is facilitated by the ability of the C-terminus of ATX1 to bind directly to Ser5P Pol II [18*]. Yeast Set1 also depends on Ser5P Pol II for its accumulation at the 5′-ends of the genes but, in contrast to ATX1, the interaction between Set1 and the CTD is indirect mediated by the Pol II-associated factor (Paf1) complex [16*].

Collectively, the results indicated that ATX1 occupancy at promoters determines the levels of TBP/Pol II (PIC) assembled at promoters regulated by ATX1, but it is Ser5P Pol II levels accumulated at the to the +300 nt position of genes that determine the amount of ATX1 recruited to this position. On its part, ATX1 is responsible for the recruitment AtCOMPASS to ATX1 regulated genes, which is essential for the generation of the H3K4me3 marks at the 5′-ends of these genes [15**]. Ultimately, these factors are involved in the transition to the elongating phase of transcription.

H3K4me3 is required for efficient elongation of transcription

The phosphorylation of serine 2 (Ser2P) of the Pol II CTD is a critical step in the process signaling the release of the Pol II into productive elongation [28**,29*]. Ser2 phosphorylation is mediated by a Ser5P-dependent mechanism [30] suggesting that both Ser2P and Ser5P modifications occur after PIC formation. For most eukaryotic genes the formation of PIC is considered the rate-limiting step of transcription (rev. in [26**]). However, the *atx1::ATX1-setm* mutants (representing catalytically disabled ATX1 protein generated by point mutations in the SET domain) displayed a different rate-limiting step. In addition to attenuated H3K4me3 levels at the 5′ ends of ATX1- regulated genes, the transcript levels, transcription rates, and Ser2P Pol II levels (indicative of the elongating form of Pol II) were all low in the *atx1::ATX1-setm* background. Surprisingly, however, the Ser5P Pol II levels at the genes' TSSs were only slightly reduced (90–85% of wild-type levels) (fig. 2).

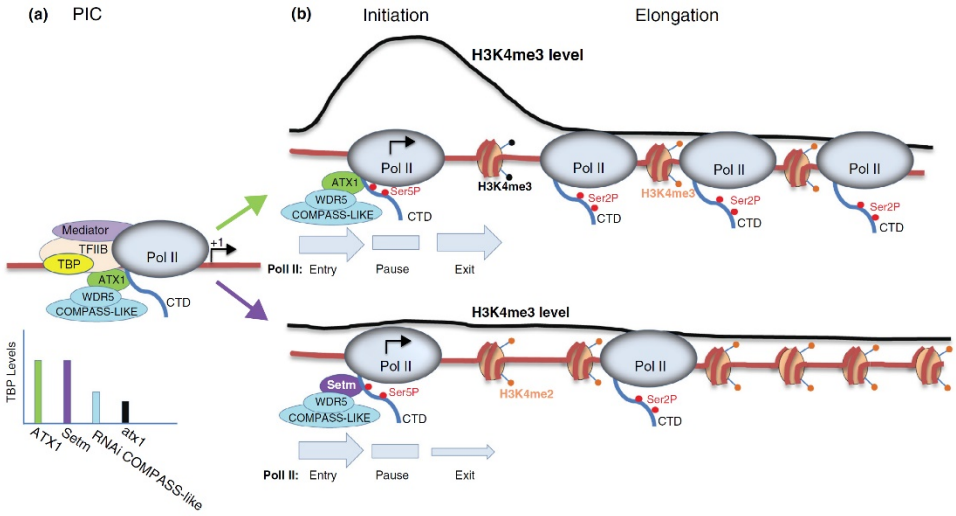

Figure 2. Model for the roles of ATX1/AtCOMPASS-like and H3K4me3 in transcription (from Ding et al., in PLoS Genet. 8(12):e1003111. http://dx.doi.org/10.1371/journal.pgen.1003111). (a) The levels of PIC assembled at promoters depend on the integrity of ATX1/AtCOMPASS-like as ATX1 occurs in a complex with TBP (yellow) and interacts with the carboxyl terminal domain of Pol II (CTD, blue tail). The bar graph in A below the template shows the relative TBP levels that occur in wild type ATX1 (green), in the ATX1-setm mutant (Setm, in purple), in RNAi COMPASS-like knockdowns (blue), and in atx1 (black) backgrounds. (b) The transition to transcription initiation and promoter proximal pausing (gray pause rectangle) occurs with similar efficiencies (gray Pol II entry arrows of similar width) for both complexes that differ only in their ATX1 subunits: wild type ATX1 (top green ATX1) and ATX1-setm mutant (bottom purple Setm). Both wild type and the ATX1-setm ATX1/COMPASS-like complexes are recruited to this site via ATX1's affinity for the Ser5P form of Pol II. Wild type ATX1/COMPASS-like produce normal amounts of H3K4me3 levels, but H3K4me3 levels are diminished in the ATX1-setm mutant (the black line above the templates shows H3K4me3 profile). The levels of H3K4me3 affect the rate of Pol II exit from the promoter proximal pause region (size of gray Pol II exit arrows below the templates), with a higher exit rate for the wild type template. These different exit rates lead to more Pol II Ser2P complexes active in transcription elongation in ATX1 than in the ATX1-setm mutant.

We proposed that the rate-limiting step for these genes was downstream of transcription initiation and that diminished H3K4me3 levels resulted in an impaired transition to elongation [15**]. This creates a form of a "stalled" Pol II accumulating at the 5′-ends in these genes providing a potential regulatory point for ATX1/AtCOMPASS dependent plant genes.

Therefore, the activating role of H3K4me3 is primarily to facilitate the transition of the Pol II into the elongation phase. Deficient levels of H3K4me3 due to depletion of Set1/COMPASS have been linked to transcriptional elongation in yeast [31] as well as to the *hsp70* locus in *Drosophila* when dSet1/COMPASS levels were reduced [17*].

Developmentally or heat-shock regulated genes in animals have paused/stalled Pol II at their 5′-ends before active transcription and require stimulation by additional factors to

move into active elongation [32]. The ATX1/AtCOMPASS regulated genes do not carry pre-accumulated Pol II at their 5′-ends but rather experience Pol II accumulation at their TSS and reduced elongation rates (as a form of stalling) when H3K4me3 levels are depleted. Stalled Pol II at yeast or plant genes in wild type backgrounds has not been reported. An interesting exception, however, are the *Arabidopsis* dehydration stress response memory genes, which retain high levels of Ser5P Pol II and H3K4me3 at their TSS and low-transcription rates during water recovery phases as "memory" marks from the previous actively transcribed phase [33]. Retention of higher H3K4me3 levels at genes after transcriptional activity has been observed also at Set1 regulated yeast genes [16*].

How does H3K4me3 affect elongation?

Our data support a model wherein ATX1 is recruited first to the promoter of an ATX1-regulated gene and then to +300 nt position of ATX1-dependent genes by directly binding to the Ser5 phosphorylated CTD of Pol II. The inability of ATX1 to bind the Ser2P modified CTD of the Pol II tail provides a plausible mechanism for ATX1's dissociation from the elongating transcription complex [18*]. ATX1 remains at the 5′-end of the genes ~300 nt downstream of the TSSs, establishing the characteristic H3K4me3 peaks at transcriptionally active genes [34]. The events at the TSS are connected with the transition to elongation and, thus, mediate the overall efficiency of transcription (rev. in [35]). However, how histone marks restricted to promoter-proximal nucleosomes activate the process downstream remains to be established. Presumably, H3K4me3 generates a chromatin environment at the 5′-end that ensures optimal elongation by recruiting pre-mRNA processing and elongation factors to the 5′ regions of [36,37,38]. H3K4me3 marks may provide binding modules for PHD domains or chromodomains present in ATP-dependent chromatin remodelers, in histone modifiers involved in acetylation, deacetylation, and demethylation [36,39]. In addition, proteins binding the H3K4me3 modification may recruit factors facilitating the elongation, like components of the spliceosome, and/or proteins involved in mRNA capping and stability [3**,23].

How do KMTs/COMPASS find their targets?

The mechanism(s) by which KMTs are recruited to their specific target genes is another important but still unanswered question. Four possible mechanisms have been proposed: (1) recruitment by sequence specific DNA binding factors; (2) direct association with the basal transcriptional machinery; (3) recruitment by RNA; and (4) association with chromatin through histone modification "readers" [5,23].

Our results with ATX1/AtCOMPASS are consistent with options (1) and (2), leave open the possibility of (3), but do not support option (4). Thus, our data compellingly indicate that ATX1/AtCOMPASS is associated directly with the basal transcriptional machinery influencing the levels of TBP and Pol II at the promoters (option 2). However, this option implies the need for a sequence-specific DNA binding factor to recruit ATX1/AtCOMPASS to the promoters (options 1 and 3). Furthermore, the absolute requirement for Ser5P Pol II

to recruit ATX1/AtCOMPASS to the TSS/+300 nt region indicates involvement of other chromatin marks in the recruitment of ATX1/AtCOMPASS (option 4) is unlikely.

We favor the view that the initial recruitment is gene specific, is mediated by specific transcription factors and/ or RNAs, and that ATX1 and AtCOMPASS are critical components of the basal transcriptional mechanism contributing to the formation/stabilization of the PIC at ATX1-regulated genes. Most importantly, ATX1/AtCOMPASS association with chromatin is a secondary recruitment event resulting from the direct binding of ATX1-SET domain to the Ser5P Pol II. MLLs, and ySet1 also associate with Ser5P Pol II but the interaction, apparently, is indirect [18*,31,40].

Similarities and differences in the molecular machinery establishing the H3K4me3 marks in yeast, animals, and *Arabidopsis*

The nearly universal distribution of the H3K4me3 mark with transcriptionally active eukaryotic genes suggests that the molecular machinery establishing the H3K4me3 marks is ancient and evolutionarily conserved. The two main components of this machinery, the KMTs and the COMPASS/COMPASS-like complexes, display features that are highly conserved in yeast, animal, and plant systems but also carry features that have significantly diverged. Phylogenetic analysis of the SET domains of the histone H3K4me3 methylating proteins revealed the KMTs belong in two distinct subfamilies: the SET sub-family and the TRX subfamily, the principle structural difference being the presence of multiple architectural domains conserved in the TRX subfamily members (fig. 1). A combinatorial assembly of various peptide domains, together with the multiplicity of TRX proteins of both animal and plant origin, generates possibilities for diversification and specialization of function. It is logical, then, to expect that the molecular mechanisms involving specific KMTs and associated complexes would be also specific to meet and accommodate functional divergences.

Three major differences between members of the SET and TRX subfamilies are that, *first*, the methyltransferases of the SET subfamily operate more globally across the genome, while the TRX-related KMTs are gene-specific [12,40,41]. Gene specificity and/or functional diversification of TRX KMTs provide a plausible reason and explanation for the multiple gene copies retained in the genomes of metazoans and plants; *second*, recruitment, histone modifications and transcription initiation of yeast genes by ySet1 may follow paths that are different from those of animal or plant genes involving TRX subfamily members. For example, monoubiquitylation of H2B (H2Bub) is a prerequisite for histone H3 lysine 4 (H3K4) di-methylation and tri-methylation by Set1 in yeast [42], while in humans dependence of H3K4me3 on H2Bub depends on the state of cell differentiation [43]. H2Bub stimulates H3K4 methylation in human cells [44] but H2Bub controls Pol II transcription elongation independently of histone H3 methylation [45]. Furthermore, H2B ubiquitylation is not required for H3K4 tri-methylation in tetrahymena [46] and we found that H3K4 tri-methylation by ATX1 also does not dependent on H2Bub (unpublished results); *third*, although the KMTs from both the SET and TRX subfamilies exhibit enzyme activity only when operating within the COMPASS/COMPASS-LIKE complexes, three subunits of which are highly conserved and indispensable for the enzyme function [37,47], the manner

in which the KMTs integrate within the complex may be different. Thus, both ATX1 and MLL1 interact with the WDR5 COMPASS subunit via a conserved peptide upstream of their SET domains, the Win motif [15**,48,49]. However, Set1 and other members of the *Arabidopsis* ATX family do not have the conserved Win domain suggesting their interaction with the COMPASS subunits differs from the Win-mediated binding of MLL1 and ATX1.

Another interesting feature relating ATX1 and MLL1 is that the two proteins have similar architectural domains in their C-terminal halves but differ in the structures of their N-half regions; in particular, the PWWP domain is missing in MLL1 (fig. 1). Remarkably, however, MLL1 critically depends on the PWWP domain for its function on chromatin, which is provided by the interacting protein LEDGF [50*]. Thus, the functional association of the LEDGF-PWWP with MLL at *Hox* genes mimics the naturally occurring arrangement of a PWWP domain at the N-terminus of the plant trithorax homologs, providing an evolutionary support for a functional link between a KMT and a PWWP. The molecular function of PWWP, however, is unknown [51].

Thereby, in some aspects, the *Arabidopsis* ATX1/AtCOMPASS displays similarities with both yeast Set1 and mammalian MLL1 models, while in other aspects the plant ATX1/AtCOMPASS complex appears distinct from the yeast or the mammalian models. Thus, enzyme activities of Set1, Trx, MLLs, and ATX1 depend on the COM-PASS/COMPASS-like complexes, three subunits of which are highly conserved. The KMTs recruit the COMPASS complexes to target genes but not vice versa [15**,16*], and the KMT/COMPASS complexes interact with Ser5P Pol II at the TSSs. However, direct interaction between the SET domain and the Ser5P CTD of Pol II has been demonstrated only for ATX1 [18*]. Furthermore, although ATX1 affects the formation/stability of the PIC (as shown also for MLL1), H3K4me3 presence is not required at ATX1/AtCOMPASS regulated promoters, in a major contrast to MLL1 [25**]. The situation at the yeast promoters appears similar to the downstream steps of ATX1 recruitment, as Set1/COMPASS is recruited to the TSS by the activated (Ser5P) Pol II after the initiation of transcription, implying H3K4me3 does not play a critical role during the assembly of the PIC. In a further similarity of events including H3K4me3 binding at the promoters, it is noted that the yeast TAF3 subunit lacks a PHD domain (required for the binding to H3K4me3) [52] and the gene encoding a TAF3 homolog is missing in *Arabidopsis* [53].

Conclusions

High H3K4me3 levels of modified nucleosomes at the 5′-ends of actively transcribed eukaryotic genes are often interpreted as evidence for a transcription activating function of this modification. However, the causative link between H3K4me3 accumulation and activation has been difficult to establish because the KMTs and COMPASS complexes may play roles beyond their catalytic function. These distinct but overlapping effects were untangled by using an experimental system where only the enzyme activity establishing the H3K4me3 marks was disabled, while preserving other potential functions. By this approach it was demonstrated that: first, ATX1/AtCOMPASS have a role at the promoters as co-activators of the basal transcriptional machinery; second, this role in transcription initiation is independent of their enzyme activity; third, the H3K4me3 marks are an essential

requirement for the transition to efficient elongation. How exactly H3K4me3 facilitates this transition into transcription elongation remains to be established.

The functions of the *Arabidopsis* ATX1/AtCOMPASS show some similar features with the yeast and mammalian models, while in other aspects ATX1/AtCOMPASS displays differences from either the yeast or the mammalian models. To our knowledge, dual roles have not been reported for the yeast Set1/COMPASS or mammalian MLL1/COMPASS-like. Thus, it is not clear whether similarities in the molecular mechanisms for gene regulation and the role of H3K4me3 extend beyond the structural similarities and the requirement for COMPASS/COMPASS-like for the enzyme activities of the KMTs. Establishing whether ySet1/COMPASS, MLL/COMPASS-like and H3K4me3 play similar roles during the specific phases of the transcription process (promoter initiation, promoter clearance, and elongation) would be of general importance for understanding the molecular mechanisms of H3K4me3-activated genes in eukaryotes.

The separate roles for ATX1/AtCOMPASS at the promoters and at the transcribed sequences downstream provide, to our knowledge, the first example of a functional role of a histone methyltransferase complex independent of its enzyme activity. We believe this paper might stimulate interest in distinguishing the roles of chromatin modifiers and their products in the specific phases of the transcription process and a critical evaluation of the role of the H3K4me3 in the transcriptional activation of plant genes.

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