The mixed-lineage leukemia fusion partner AF4 stimulates RNA polymerase II transcriptional elongation and mediates coordinated chromatin remodeling

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AF4 gene, frequently translocated with mixed-lineage leukemia (MLL) in childhood acute leukemia, encodes a putative transcriptional activator of the AF4/LAF4/FMR2 (ALF) protein family previously implicated in lymphopoiesis and Purkinje cell function in the cerebellum. Here, we provide the first evidence for a direct role of AF4 in the regulation of transcriptional elongation by RNA polymerase II (Pol II). We demonstrate that mouse AF4 functions as a positive regulator of Pol II transcription elongation factor b (P-TEFb) kinase and, in complex with MLL fusion partners Af9, Enl and Af10, as a mediator of histone H3-K79 methylation by recruiting Dot1 to elongating Pol II. These pathways are interconnected and tightly regulated by the P-TEFb-dependent phosphorylation of Af4, Af9 and Enl which controls their transactivation activity and/or protein stability. Consistently, increased levels of phosphorylated Pol II and methylated H3-K79 are observed in the ataxic mouse mutant robotic, an over-expression model of Af4. Finally, we confirm the functional relevance of Af4, Enl and Af9 to the regulation of gene transcription as their over-expression strongly stimulates P-TEFb-dependent transcription of a luciferase reporter gene. Our findings uncover a central role for these proteins in the regulation of transcriptional elongation and coordinated histone methylation, providing valuable insight into their contribution to leukemogenesis and neurodegeneration. Since these activities likely extend to the entire ALF protein family, this study also significantly inputs our understanding of the molecular basis of FRAXE mental retardation syndrome in which FMR2 expression is silenced.

INTRODUCTION

The gene encoding AF4 is one of the most common of over 30 fusion partners of the mixed-lineage leukemia (MLL) gene in 11q23 chromosomal translocations associated with particularly aggressive forms of childhood acute leukemia (1–3). Together with the much rarer but recurrent MLL fusion partners LAF4 and AF5q31, and the fragile X-mental retardation 2 (FMR2) protein of nuclear proteins (4–7). These share a common domain organization including N- and C-terminal homology domains of unknown function, a highly conserved serine-rich transactivation domain (TAD) and the ALF homology domain which we recently showed promotes degradation through the proteasome pathway by mediating interaction with SIAH ubiquitin ligases (7–14). As for numerous intracellular proteins (15), the proteasome regulates the stability and turnover of the ALF proteins, thus ensuring tight control of their biological function(s). Although AF4 has been studied for over a decade, aspects of its normal function have only recently emerged with loss-of-function and gain-of-function mouse mutants. The knockout mouse which shows severe impairment in the maturation of B- and T-cell populations first revealed a critical role for AF4 in lymphoid development (16), the disruption of which is suspected to underlie the lymphocytic nature of leukemias associated with the t(4;11)(q21;q23) translocation. More recently we identified Af4 as the disease gene in the robotic mouse, a dominant ENU mutant which in addition to defects in early T-cell maturation develops ataxia

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following Purkinje cell degeneration in the cerebellum, uncovering a totally unexpected role for AF4 in Purkinje cell function essential for the control of balance and motor coordination (17,18). Further analysis revealed that the robotic mutation (V280A), which lies in the ALF domain, disrupts a conserved residue of the consensus binding motif for SIAH proteins, resulting in significantly reduced (~2–3-fold) proteasome-dependent turnover of mutant AF4 in transfected mammalian cells (14). The robotic mouse thus potentially represents a valuable over-expression model in which to study the functional contribution of AF4 to lymphopoiesis and Purkinje cell physiology. The transcriptional activity and underlying molecular mechanisms that mediate these functions also remain to be characterized. In the absence of sequence-specific DNA binding capacity, AF4 and members of the ALF family are thought to function as co-activators by linking DNA-binding transcription factors to the basal transcriptional machinery or by modifying components of the transcriptional apparatus and/or chromatin factors that repress or facilitate gene expression (19).

Recently, the activity of the RNA polymerase II (pol II) elongation factor eleven–nineteen lysine-rich in leukemia (ELL), also frequently fused to MLL in childhood leukemia, was shown to be strongly stimulated by ELL-associated factors (EAF) 1 and 2 (20–22). Interestingly, the EAF proteins contain a TAD with some homology to that of the ALF proteins, suggesting that the latter may also associate with and positively regulate factors with similar functions. Consistent with this postulate, AF5q31 had previously been co-purified from mammalian cells with the positive transcription elongation factor b (P-TEFb), a complex of cyclin-dependent kinase 9 (CDK9) and cyclin T1, which overcomes transcriptional pausing imposed by DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF), enabling productive elongation to proceed (12,23). The functional significance of the AF5q31/P-TEFb complex, however, was never investigated.

Covalent modifications of histone tails play fundamental roles in chromatin structure and function. In particular, methylation of histone 3 (H3) has emerged as a key regulator of gene expression during transcriptional elongation, and recent efforts have focused on understanding the molecular basis of H3 methylation at K4, K36 and K79 that typically mark actively transcribed chromatin regions (24,25). Several studies have established that MLL and SET2, the mammalian H3-K4 and H3-K36 methyltransferases, are recruited to their chromatin substrates by pol II-associated factor (PAF) which mediates their association with phosphorylated Pol II (24,26–29). In contrast, the mechanism by which the H3-K79 methyltransferase DOT1 is recruited to elongating Pol II remains to be identified. DOT1 was recently found to be a H3-K79 methyltransferase DOT1 is recruited to elongating Pol II remains to be identified. DOT1 was recently found to be a H3-K79 methyltransferase that is recruited to elongating Pol II, through phosphorylation of Pol II C-terminal domain (CTD) and DSIF, enabling productive elongation to proceed (12,23). The functional significance of the AF5q31/P-TEFb complex, however, was never investigated.

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RESULTS

AF4 associates with P-TEFb and stimulates its kinase activity

AF5q31 was previously purified from HeLa nuclear extracts in complex with P-TEFb and co-immunoprecipitation experiments mapped the CDK9- and Cyclin T1-binding regions to the amino terminal (1–420) and central (420–715) parts of the protein, respectively (12). As these regions share over 50% similarity (35–38% identity) with the corresponding sequences of AF4, we tested the possibility of a similar interaction between mouse AF4 and P-TEFb in HEK293T cells co-expressing HA-tagged AF4, Cdk9 and Cyclin T1. Antibodies against each protein could indeed efficiently co-immunoprecipitate the other two, demonstrating a stable association between AF4 and P-TEFb (Fig. 1A). An in vitro binding assay further established that, as for AF5q31, this interaction is mediated by both the kinase and cyclin subunits of P-TEFb (Fig. 1B).

To determine whether this association had an effect on P-TEFb kinase activity, phosphorylation of recombinant Pol II CTD was examined in vitro in the presence or absence of AF4. As shown in Figure 2A, addition of AF4 caused a dramatic increase in the levels of phosphorylated Pol II CTD (Pol IIo), revealing a highly potent stimulatory effect towards P-TEFb kinase activity. Consistently, a large increase in Pol IIo levels was observed in HEK293T cells overexpressing P-TEFb in the presence of AF4 (Fig. 2B). Phosphorylation of the negative Pol II elongation factors DSIF (p160 subunit) and NELF (E subunit) by P-TEFb was also significantly enhanced in the presence of AF4 in vitro (Fig. 2C).

In agreement with the previously established transcriptional activation capabilities, these data identify AF4 as a positive regulator of P-TEFb kinase activity and elongation function, and thus as a positive regulator of Pol II transcriptional elongation.
P-TEFb phosphorylates Af4 and down-regulates its transactivation activity

Af4 is typically detected as a doublet composed of the full-length protein (~183 kDa) and what most likely represents a stable degradation product (~143 kDa) (14). Intriguingly, the Af4 doublet displayed a reproducible decrease in electrophoretic mobility when the protein was co-expressed with P-TEFb (Fig. 1A), suggesting that this phenomenon could be accounted for by phosphorylation. Using an in vitro kinase assay, we showed that P-TEFb indeed phosphorylates Af4, resulting in an increase in molecular weight of ~10 kDa (Fig. 3A). P-TEFb-mediated phosphorylation of Af4 was subsequently confirmed in HEK293T cells (Fig. 3B). As expected, phosphorylation of Af4 was abolished by cell treatment with DRB, an inhibitor of P-TEFb kinase activity (middle panel) (39). Finally, in vitro de-phosphorylation of the 193 kDa protein band with calf intestinal alkaline phosphatase (CIP) resulted in a shift in size down to 183 kDa (right panel).

We have previously established that Af4 is targeted for rapid degradation by the proteasome via its interaction with Siah-1a and Siah-2 ubiquitin ligases (14). Since the degradation of a number of protein targets by the proteasome system is regulated by their phosphorylation state (40), we reasoned that phosphorylated Af4 might be resistant to Siah-mediated degradation by the proteasome. To test this hypothesis, HEK293T cells were co-transfected with Af4 and Siah-1a in the presence or absence of P-TEFb and Af4 protein levels were analyzed at 12 h post-transfection, a time point that corresponds to the half-life of the protein (14) (Fig. 3C). At 12 h, Af4 levels were already significantly reduced in the cells co-expressing Siah-1a (lane 2); degradation of phosphorylated Af4, however, occurred at a similar rate (lane 3). By 24 h, proteasomal degradation of both phosphorylated and un-phosphorylated forms of Af4 was complete. Contrary to our expectations, these results clearly excluded phosphorylation as a modulator of Af4 stability.

Phosphorylation-induced conformational changes control the catalytic activity of numerous proteins. Based on a previously established transactivation CAT ELISA assay in HeLa cells (14), we next investigated whether phosphorylation of Af4 modified its transcriptional activation potential. As shown in Figure 3D, co-expression of P-TEFb with Af4 resulted in a dramatic decrease in the expression of the CAT reporter gene. Taken together, these results identify Af4 as a new substrate of P-TEFb kinase and argue that phosphorylation of Af4 negatively regulates its transcriptional activation activity.

AF9 and Eln protect Af4 from degradation by the proteasome

Recent studies have reported the association of human AF4 with the YEATS family homologues AF9 and ENL (37,38). Using co-immunoprecipitation, we first confirmed the interaction between mouse HA-Af4 and each of FLAG-Af9 and Myc-Enl in transfected HEK293T cells (Fig. 4A). Given that the binding domains for Siah (amino acids 273–289) and AF9/ENL (amino acids 766–779) are located within distinct regions of AF4, we tested whether the latter could accommodate both interactions at once. As seen in Figure 3C, co-expression of Siah-1a with Af4 led to its proteasomal degradation (Fig. 4B, lane 2). Unexpectedly, additional co-expression of Enl (lane 3) or Af9 (lane 4) stabilized Af4 protein levels, indicating that the binding of Enl and Af9 to Af4 displaces that of Siah-1a. These results thus importantly identify a mechanism of allosteric interference that enables Af4 to escape Siah-mediated degradation by the proteasome when in complex with Af9 or Enl.

Phosphorylation of AF9 and ENL by P-TEFb promotes their proteolysis

As for Siah-1a, we investigated whether the interactions of Af4 with P-TEFb (Fig. 1A) and with Enl or Af9 (Fig. 4A) were mutually exclusive by co-expressing all four proteins in HEK293T cells. In addition to Af4, antibodies directed against Myc-Enl and FLAG-Af9 efficiently co-immunoprecipitated the Cdk9 and Cyclin T1 subunits of P-TEFb (Fig. 4C),
demonstrating the co-existence of P-TEFb, Af4 and one of the Enl or Af9 homologues in a single protein complex. Given the transactivation capabilities of Enl and Af9, we also examined the possibility that their binding to Af4 may amplify further its stimulatory effect on P-TEFb kinase activity. Co-expression of Enl or Af9 with P-TEFb and Af4 in HEK293T cells, however, had no additional effect on the levels of Pol II CTD phosphorylation previously observed in Figure 2B (data not shown).

Surprisingly, four times more starting protein material had to be used to immunoprecipitate Enl and Af9 when co-expressed with P-TEFb (Fig. 4C) than without (Fig. 4A). As such a decrease in their expression was unlikely to be solely accounted for by the additional transfection of the two constructs encoding P-TEFb subunits, we performed a direct comparison of Enl and Af9 protein levels when co-expressed with Af4 in the presence or absence of P-TEFb (Fig. 4D). Although relatively high levels of Enl and Af9 could be immunoprecipitated in cells co-expressing Af4 (lane 1), their detection was completely abolished from cells also co-expressing P-TEFb (lane 2). To confirm that the disappearance of Enl and Af9 was associated with P-TEFb kinase activity, cells were further treated with the Cdk9 kinase inhibitor DRB; this treatment indeed significantly rescued Enl and Af9 protein levels (lane 3). Demonstrating a direct interaction between P-TEFb and Enl or Af9, we subsequently showed that P-TEFb phosphorylates the latter in vitro (Fig. 4E, lane 2) in a reaction strongly stimulated by Af4 (lane 3). As discussed above, phosphorylation promotes the proteasome-dependent degradation of a number of protein substrates. To determine whether phosphorylated Enl and Af9 are targeted for proteasomal degradation, HEK293T cells co-expressing Enl or Af9 with P-TEFb were treated with the proteasome inhibitor MG132 (Fig. 4F). On the contrary, these conditions enhanced the proteolysis of Enl and Af9, possibly via the stabilization of endogenous Af4 protein levels which in turn stimulate further P-TEFb-mediated phosphorylation of Enl and Af9. These data thus provide evidence that P-TEFb-mediated phosphorylation of Enl and Af9 promotes their degradation.

P-TEFb, Af4, Enl/Af9 and Af10 form a complex that links elongating Pol II to H3 and mediates DOT1-dependent methylation of H3-K79

Human ENL was also recently isolated as a binding partner of AF10 and DOT1 (38). Interestingly, direct interactions between AF10 and DOT1, and ENL (YEATS domain) and H3 were concomitantly reported (30,38). Given that distinct regions of AF10 are engaged in the binding with ENL (amino acids 206–350) and DOT1 (amino acids 719–800), we investigated the possibility that these proteins may form a stable complex in vivo. As shown in Figure 5A, mouse Af10, Dot1 and Myc-Enl could indeed be co-precipitated from transfected HEK293T cells using an anti-Myc antibody.
As anticipated, we could also demonstrate similar interactions with Dot1, Af10 and the Enl homologue Af9 (lane 2). Given that Enl and Af9 were also found to interact with P-TEFb and Af4 (Fig. 4C), we next addressed the possibility that the YEATS homologues may bring all these protein partners into the same complex, and first examined their ability to simultaneously bind Af4, Af10 and Dot1. When co-expressed in HEK293T cells, the two sets of four proteins indeed formed complexes whose subunits could be precipitated using an anti-HA antibody (Fig. 5A, lanes 3 and 4). The existence of a large complex integrating P-TEFb, Af4, Af10, Dot1 and one of the Enl or Af9 homologues was subsequently confirmed by successfully precipitating methylated H3-K79 (mH3-K79) using the anti-CDK9 antibody (Fig. 5A, lanes 5 and 6). These results provide strong evidence that this complex links elongating Pol II to H3, thus identifying a potential mechanism by which Dot1 may be recruited to actively transcribed genes.

To confirm the role of this complex in mediating methylation of H3-K79, we examined the levels of mH3-K79 in HEK293T cells over-expressing one or more of its protein components (Fig. 5B). As expected, over-expression of Dot1 led to a marked increase in H3-K79 methylation (lane 2). In agreement with the above, over-expression of Af4 (lane 3), Enl (lane 5), Af9 (lane 7) and Af10 (lane 8) was similarly associated with a significant increase in mH3-K79 levels. This result also importantly revealed that the stimulating effect of each complex subunit is not limited by the availability of the others; however, these are not cumulative (see lane 9) and thus still ultimately depend on the available levels of Dot1. Similarly, no significant cumulative effect was observed between over-expression of Dot1 and

Figure 3. P-TEFb phosphorylates Af4 and controls its transactivation activity. (A) In vitro Af4 kinase assay. HA-Af4 was incubated in the presence or absence of P-TEFb and DRB (10 μM) in reactions containing [γ-32P]ATP, and analyzed by autoradiography. (B) In vivo analysis of Af4 phosphorylation. HEK293T cells over-expressing HA-Af4 alone or in combination with P-TEFb were treated in the presence or absence of DRB (100 μM). Corresponding protein lysates were immunoprecipitated with an anti-HA antibody, and immunoprecipitates were treated in the presence or absence of calf intestinal alkaline phosphatase (CIP), and analyzed by western blot using the anti-HA antibody. (C) Effect of Af4 phosphorylation on Siah-1a-mediated degradation by the proteasome. HEK293T cells were transfected with constructs encoding HA-Af4, Siah-1a and P-TEFb for 12 or 24 h. Corresponding protein lysates were immunoprecipitated with an anti-HA antibody and analyzed by western blot using the indicated antibodies. β-tubulin detection shows equal inputs for IP. (D) CAT ELISA transactivation assay. (Top panel) HeLa cells were transfected with the CAT reporter construct pG5CAT and pM (1) or pM-Af4 encoding Gal4-DNA binding domain fusion proteins (2), in the presence (3) or absence (2) of pCDNA3 constructs encoding P-TEFb, and CAT amounts present in the corresponding protein lysates were measured by ELISA. Results are shown ±SD; n = 3. (Bottom panel) RT–PCR analysis using primers specific for GAPDH, CAT, Gal4-Af4, Cdk9 and Cyclin T1 (Cyc T1) shows equivalent cell transfection efficiency and in vivo transcription of the transfected constructs in samples 1–3. Products were resolved on a 1% agarose gel along with 100 bp and 1 kb ladders (lanes M1 and M2). Note that only full-length HA-Af4 is shown in (A), (B) and (C), and that pM-Af4 used in (D) only contains amino acids 1–515 of Af4 cDNA.
Figure 4. P-TEFb, Af4 and Af9/Enl form a complex that leads to the stabilization of Af4 and degradation of Enl/Af9. Protein lysates from HEK293T cells over-expressing the indicated proteins were immunoprecipitated and analyzed by western blot using the indicated antibodies. β-tubulin detection and RT–PCR analysis show equal inputs for IP and equivalent transfection efficiency and in vivo transcription of the transfected constructs, respectively. (A) Af4 associates with Enl and Af9. (B) Binding of Enl and Af9 protects Af4 from Siah-1a-mediated degradation by the proteasomal. (C) P-TEFb, Af4 and Enl/Af9 co-exist in the same complex. Cells were co-transfected with constructs encoding P-TEFb (Cdk9 and Cyclin T1), HA-Af4 and Myc-Enl (lane 1) or FLAG-Af9 (lane 2). (D) P-TEFb kinase activity is associated with the degradation of Enl and Af9. Transfected cells were further treated in the presence or absence of DRB. The results shown for co-immunoprecipitated HA-Af4, Cyclin T1 and Cdk9 are those obtained with the anti-Myc antibody; similar results were obtained with the anti-FLAG antibody. (E) P-TEFb phosphorylates Enl and Af9 in vitro. Phosphorylation of Enl and Af9 by P-TEFb was examined using an in vitro kinase assay as in Figure 2. (F) P-TEFb-mediated degradation of Enl and Af9 does not occur through the proteasome pathway. Transfected cells were further treated in the presence or absence of the proteasome inhibitor MG132 (10 μM).
components of the complex such as Af4 or Enl (compare lanes 4 and 6 with lane 2), making unlikely the possibility that in addition to their role in recruiting Dot1, Enl and Af9 also stimulate its methyltransferase activity. Finally, consistent with P-TEFb-mediated proteolysis of Enl and Af9 (Fig. 4F), co-expression of P-TEFb with Af4 and Enl or Af9 brought mH3-K79 back to basal levels (Fig. 5C, lanes 4–6). Taken together, these data support a potential negative feedback model for the regulation of H3-K79 methylation whereby P-TEFb first promotes the recruitment of Af4, Enl/Af9 and Af10 and ultimately Dot1 to Pol II elongation complex, and subsequently the phosphorylation-dependent proteolysis of Enl/Af9 which results in the loss of Dot1 recruitment.

Increased Pol II CTD phosphorylation and H3-K79 methylation occur in the robotic mouse, an over-expression model of Af4

Previous functional characterization of the robotic mutation in transfected HEK293T cells predicted abnormal accumulation of Af4 in Purkinje cells of the robotic mouse cerebellum and other sites of normal expression due to a deficit in proteasome-dependent degradation (14). To verify this postulate, a polyclonal antiserum was raised against a central region of Af4 that shared minimal homology with the other family members. Since expression of Af4 is particularly low in the brain and specific to Purkinje cells, the analysis was conducted in thymus where levels are about 100-fold higher (41). The Af4 antiserum detected a specific protein band of ~193 kDa the intensity of which was 2–3-fold stronger in the robotic sample when compared with wild-type (Fig. 6), concomitantly revealing that Af4 predominantly exists under its phosphorylated inactive form (see Fig. 3). In addition, the levels of Siah-1 protein co-immunoprecipitated with robotic Af4 were

Figure 6. Af4 abnormally accumulates in the robotic mouse. Thymus protein homogenates from 5-weeks old wild-type (Wt) and robotic (Rob) mice were immunoprecipitated and analyzed by western blot using the Af4 antiserum (immune) or anti-SIAH-1 antibody. To verify the specificity of the signal detected by the Af4 antiserum, the membrane was stripped and re-probed with the pre-immune serum. β-tubulin detection shows equal inputs for IP. The asterisk indicates a non-specific band previously described for this antibody (14).
significantly reduced when compared with wild-type (Fig. 6). These data were consistent with, and quantitatively matched, those previously obtained in HEK293T cells, thus confirming stabilization and accumulation of the mutant protein in vivo and validating the use of the robotic mouse as an over-expression model of Af4.

To confirm the previously established role of Af4 as a positive regulator of P-TEFb kinase activity (Fig. 2) and mediator of H3-K79 methylation (Fig. 5), the levels of Pol II CTD phosphorylation and H3-K79 methylation were examined in the cerebellum and thymus of robotic and wild-type animals (Fig. 7A). In agreement with the above, an ~2-fold increase in the levels of Pol II, and mH3-K79 was observed in the robotic cerebellum when compared with wild-type (left panels). Surprisingly, while increased methylation of H3-K79 also occurred in the robotic thymus, the levels of Pol II, were unaffected in this tissue (right panels), potentially revealing differential transcriptional regulatory roles of Af4 in the cerebellum and thymus or a compensatory mechanism to maintain normal Pol II levels in the thymus. Consistent with these results, the Af4 antiserum pulled down about twice as much Af4 (as seen in Fig. 6), P-TEFb (Cyclin T1 and Cdk9), Af10, Dot1 and mH3-K79 from robotic thymus protein homogenates as compared with wild-type, while similar amounts of Pol II CTD were co-immunoprecipitated (Fig. 7B, left panels). Although neither Enl nor Af9 could be detected from the Af4 immunoprecipitate, this was presumably due to multiple stripping and re-probing cycles of the membrane as an anti-ENL antibody efficiently immunoprecipitated Af4, as well as other components of the complex (right panels). The low levels of Af4 expression in the cerebellum hamper the isolation of a similar complex in this tissue; however, co-localization of these proteins with Af4 in Purkinje cells was confirmed by immunohistochemical staining of cerebellum sections (Fig. 7C).

**Af4, Enl and Af9 collaboratively stimulate P-TEFb-dependent gene transcription**

To confirm the functional contribution of Af4, Enl and Af9 to the regulation of gene expression, the effect of their
over-expression on the transcription levels of the luciferase reporter gene was monitored in neuronal N2a cells (Fig. 8); this cell type was chosen for the detectable levels of endogenous Af4 protein (data not shown). In agreement with the stimulatory effect of Af4 on P-TEFb elongation activity demonstrated earlier, a 7-fold increase in the levels of luciferase was observed in cell transfected with Af4 (Af4-wt, lanes 2 and 3). Luciferase expression was enhanced by a further 3-fold when using a stable mutant version of Af4 carrying the robotic mutation (Af4-mut, lane 4) of which Siah-1a-dependent degradation by the proteasome was consistently reduced by an equivalent factor (lanes 5 and 6), as previously observed in HEK293T cells (14). Presumably owing to the stabilization of endogenous Af4, over-expression of Enl and Af9 also resulted in a strong increase in luciferase transcription (lanes 8 and 9) which was additive to that of Af4 in co-transfected cells (lanes 12 and 13), reaching levels almost as high as those observed in cells solely over-expressing P-TEFb (lane 10). Confirming that the transcriptional activation activity of Af4, Enl and Af9 is dependent on their (direct or indirect) effect on P-TEFb activity, their co-expression with a mutant version of P-TEFb (P-TEFb-mut) composed of cyclin T1 and an inactive (D167N) form of Cdk9 (42) abolished their stimulatory transcriptional potential (lanes 17–19) to levels similar to those observed in cells transfected with P-TEFb-mut only (lane 11). Co-expression with fully functional P-TEFb completely abolished luciferase gene expression (lanes 14–16), presumably due to the negative feedback loop.

Figure 8. Af4, Enl and Af9 stimulate gene expression. (Top panel) Luciferase assay. N2a cells were transfected with (lanes 2–19) or without (mock, lane 1) pGL2 SV40-luciferase reporter construct, and in the presence (lanes 3–19) or absence [non-transfected (NT) lane 2] of pCDNA3 constructs encoding the indicated proteins. Af4-mut carries the robotic mutation (V280A). P-TEFb-mut is composed of Cyclin T1 and an inactive (D167N) form of Cdk9. Luciferase activity was measured in the corresponding protein lysates 36 h post-transfection. Results are shown ±SD, n = 3. (Bottom panel) RT–PCR analysis using primers specific for luciferase, Af4, Siah-1a, Af9, Enl, Cdk9 and Cyclin T1 shows the levels of transfection efficiency and in vivo transcription of the expressed constructs in samples 1–19.
operated by P-TEFb-dependent phosphorylation of Af4, Enl and Af9 that controls Af4 transactivation activity as well as Af9 and Enl protein stability.

DISCUSSION

In this report, we demonstrate that AF4 which is associated with leukemia in man and ataxia in the mouse is present in a transcriptional regulatory chromatin remodeling complex with AF9, ENL, and AF10, also previously implicated in leukemia and human neurological dysfunction. Our data provide evidence that these proteins collaboratively function as positive regulators of gene transcription by stimulating P-TEFb Pol II kinase activity and mediating Dot1-dependent methylation of H3-K79, thus facilitating the transition of Pol II into productive elongation and its processivity along the DNA template by maintaining the chromatin structure in an open conformation.

Through its ability to phosphorylate Pol II CTD at Ser-2, P-TEFb not only controls the productive elongation of most eukaryotic genes but also coordinates downstream events including pre-mRNA splicing and 3′end processing (43,44). Recent studies have shown that P-TEFb is sequestered into a large kinase-inactive complex through its association with 7SK RNA and HEXIM1/2 proteins (45–47). With the identification of the stimulatory activity of AF4, we uncover here an additional level of regulation of P-TEFb function. This finding importantly implies that the levels of active P-TEFb ultimately depend on the interplay between positive (AF4) and negative (7SK/HEXIM dimer) regulators competing for its binding. Other transcriptional activators including HIV Tat, CIITA, c-Myc, NFκB, MyoD and Brd4 have also been shown to stimulate transcriptional elongation through their association with P-TEFb (48–54); unlike AF4, however, no direct stimulatory effect on P-TEFb activity was reported or could be demonstrated and these proteins were found to function by mediating the recruitment of P-TEFb to specific gene promoter regions. Similarly, the restricted expression of AF4 to lymphoid organs (thymus, spleen, bone marrow) and Purkinje cells of the cerebellum (17,41) certainly provides, if not a gene-specific, at least a tissue-specific regulatory mechanism for P-TEFb function. Given the reported interaction with AF5q31 (12) and the high degree of sequence similarity between the ALF proteins within the Cdk9- and Cyclin T1-binding regions (over 42%), we anticipate that this stimulatory function also applies to the rest of the family. Supporting this assumption, we have indeed verified an interaction between P-TEFb subunits and Fmr2 (unpublished data). Most likely attributable to their homologous TAD, a similar function to the ALF proteins was recently reported for the EAF1/2 family proteins towards the elongation activity of ELL (55). The ALF and EAF proteins thus represent a novel class of homologous elongation co-factors, the stimulatory function of which is likely essential for the timely execution of both Pol II gene transcription and RNA processing.

In the past few years, the molecular mechanisms underlying chromatin remodeling through methylation at H3-K4, -K36 and -K79 that are imposed at the promoter and in early complexes (K4) or during the productive phase (K36 and K79) of transcriptional elongation have become a topic of intense interest. MLL and SET2 which, respectively, methylate H3-K4 and H3-K36 are recruited to elongating Pol II either directly (SET2) or indirectly (MLL) via association with the PAF elongation complex (27–29). Components of the PAF complex are also required for methylation of H3-K79 by DOT1, but no direct interaction between DOT1 and the PAF complex has yet been reported (27). In this study, we show that P-TEFb, Af4, Enl/Af9, Af10 and Dot1 co-exist in a single complex in mammalian cells and tissues; consistently, over-expression of these subunits was found to mediate H3-K79 methylation. Our data collectively support a model whereby following the association of P-TEFb with the AF4-ENL/AF9 complex and their recruitment to the transcription machinery, the contacting surface between AF4, ENL/AF9 and AF10 provides a ‘platform’ for the recruitment of DOT1 to elongating Pol II (Fig. 9). In this model, DOT1-dependent methylation of H3-K79 is tightly regulated by the interplay between negative (7SK-HEXIM1/2 dimer) and positive (AF4) regulators of P-TEFb kinase activity, P-TEFb-mediated down-regulation of AF4 transactivation activity, P-TEFb-mediated proteolysis of ENL and AF9, and SIAH-mediated proteosomal degradation of AF4. These regulatory loops undoubtedly ensure a very tight control of P-TEFb and AF4 activities in particular, highlighting these as critical levels of regulation in transcriptional elongation by Pol II.

We have previously identified loss of FMR2 expression in the hippocampus and amygdala as the underlying cause of FRAXE, a mild-to-borderline (50 < IQ < 85) form of mental retardation associated with learning deficits, especially delays in language acquisition, and epileptic seizures (6). Although the Fmr2 knockout mouse is now available and efficiently recapitulates the disease phenotype (56), the lack of information regarding the normal function and activity of FMR2 in cognitive processing has hampered further advances into the molecular basis of FRAXE. With this study, we provide an indirect functional characterization of FMR2 transcriptional activity the loss of which in FRAXE patients is expected to result, if not in the silencing, at least in a significant decrease in the expression of genes involved in synaptic plasticity underlying learning and memory functions. Supporting evidence includes reports of significant-to-complete loss of CDK activities in the brain of patients with Down, atypical Rett and X-linked West mental retardation syndromes associated with mild-to-profound deficits in language and communication skills (57–59), and, as seen in the Fmr2 knockout mouse, of severe impairment in context-dependent fear conditioning following inhibition of Cdk5 activity in the hippocampus, a kinase previously implicated in the regulation of long-term synaptic potentiation and suspected to function in collaboration with Cdk9 to activate differentiation-specific genes in this compartment (56,60–63). Also noteworthy is the emerging importance of epigenetic, chromatin remodeling mechanisms in synaptic plasticity and memory formation by enabling the translation of stored cellular information into behavioral memory (64).

More recently, we identified another ALF family member Af4 as the disease gene in a novel mouse model of autosomal cerebellar ataxia (17). Here, we confirm that the Purkinje cell degeneration and T-cell developmental defects observed in the
Figure 9. Model of coordination of Pol II processivity and H3-K79 methylation by P-TEFb, AF4, ENL/AF9 and AF10 during transcriptional elongation. (A) Upon formation of the pre-initiation complex composed of Pol II and the general transcription factors (GTFs), Kin28 subunit of TFIIH phosphorylates Pol II CTD on Ser-5 (S5) which initiates elongation (1). The PAF complex is recruited to phosphorylated Pol II and, by mediating the association of the Rad6/Bre1 complex, triggers ubiquitination of histone H2B at K123 creating localized disruption of nucleosomes (2). PAF recruits the MLL complex, resulting in the methylation of H3-K4 at the promoter (3). The negative elongation factors DSIF and NELF also associate with phosphorylated Pol II and concerted ly promote its arrest, ensuring 5'-capping by the capping enzyme (CE) (4). (B) P-TEFb, the activity of which depends on a reversible exchange between the stimulatory AF4-ENL/AF9 and the inhibitory 7SK/HEXIM1 complexes (6), is recruited (5) and overcomes transcriptional pausing by phosphorylating Pol II CTD on Ser-2 (S2) as well as DSIF and NELF which are then released (7). P-TEFb also phosphorylates AF4 which down-regulates its transactivation activity, providing a negative feedback mechanism for the control of P-TEFb elongation activity (8). Concomitantly, ENL/AF9 associates with histone H3 and recruits AF10 to the AF4-ENL/AF9 complex, which via interactions with both AF10 and ENL/AF9 mediates the recruitment of DOT1 to elongating Pol II and methylation of H3-K79 (9). As productive elongation proceeds and following loss of S5 phosphorylation, the MLL complex departs and is replaced by SET2 which directly interacts with S2-phosphorylated Pol II and methylates H3-K36 (10). (C) Phosphorylation of ENL and AF9 by P-TEFb (8) results in their degradation via an unknown mechanism (11). In the absence of ENL/AF9, AF4 is exposed to ubiquitination by SIAH and subsequent degradation by the 26S proteasome (12). DOT1 and SET2 continue to catalyze methylation throughout the remainder of the gene. EAF1/2 stimulates the activity of ELL (13) which together with DSIF p160 travels with Pol II enhancing its processivity. Grey barrels depict nucleosomes, and red stars indicate methylation of histone H3. Ub, ubiquitin group; P, phosphate group.
Chromosomal rearrangements found in patients with acute lymphoblastic (ALL) and myeloid (AML) leukemia fuse the N-terminal DNA binding domain of MLL in-frame to one of over 30 heterogeneous protein partners with transcriptional activation or dimerization activity (77). This invariably results in the conversion of MLL into a constitutive transcriptional activator that maintains persistent expression of a subset of its normal Hox gene targets, typically HoxA7 and HoxA9 (78–81). According to a recent study, AF4, AF9, ENL and AF10 are the four most frequent fusion protein partners of MLL in pediatric-, adult- and therapy-related acute leukemias (82). Here, we show that these proteins are also functionally linked and collaboratively regulate the productive phase of transcriptional elongation. As the interaction domains of AF4 with AF9/ENL, ENL/AF9 and AF10 with DOT1 are retained in the respective MLL-fusion proteins, our proposed model predicts that the fusion proteins will similarly mistarget DOT1 to HoxA7 and HoxA9. Consistent with this postulate, up-regulation of Hoxa9 expression is associated with increased levels of mH3-K79 in cells transformed by MLL-AF10 and MLL-ENL (30,83). While our data have demonstrated the collaborative function of a subset of MLL fusion partners in transcriptional regulation, we and others have recently become aware of the potential existence of a much larger protein network integrating MLL and up to 13 other translocation partners (18,37). Remarkably, MLL was previously isolated from a multi-protein supercomplex composed of at least 29 factors with transcriptional, chromatin remodeling, histone-acetylation, -deacetylation and -methylation, and RNA processing activities bound to the promoter region of the HoxA9 gene (84). Taken together, these findings strongly suggest that MLL-fusion proteins disrupt and/or incorrectly target a common transcriptional regulatory pathway, and raise the possibility that pharmacologic manipulation of H3-K79 methylation levels through inhibition of DOT1 methyltransferase activity could be of therapeutic benefit for at least a number of ALL and AML subtypes with extremely poor prognosis.

The characterization of the ALF family transcriptional regulatory activity presented here provides valuable information to assist future studies aimed at identifying the gene targets deregulated in ALF-associated neurodegeneration, mental retardation and leukemia. This study also raises the possibility that some components of the transcriptional regulatory protein network in which the ALF proteins function may be involved in other disorders of the central nervous system.

MATERIALS AND METHODS

Antibodies

Anti-HA, -FLAG, -c-Myc and β-tubulin-1 antibodies were obtained from Sigma, anti-D28K Calbindin from Swant, anti-SIAH-1 (N-15), -Cyclin-T1 (H-245) and -CDK9 (C-20) from Santa Cruz Biotechnology, anti-RNA Polymerase II CTD, -dimethyl-Histone H3 (Lys79) and -AF10 from Abcam, anti-MLLT1/ENL (C-term), -MLLT3/AF9 (C-term) and -DOT1L (N-term) from Abgent and -phospho-Rpb1 CTD (Ser2/5) from Cell Signalling. The Af4 antisera was produced by Eurogentec through immunization of rabbits with
a recombinant polypeptide corresponding to amino acids 395–888 of mouse Af4 cDNA sequence (GenBank accession no. AF074266).

**Plasmid construction**

Full-length cDNA sequences of mouse Enl, Af9 and Cdk9 were amplified by PCR from IMAGE clones 5716396, 5012671 and 3601310 (MRC Gene Service) respectively, and cloned into pCDNA3 (Invitrogen) in-frame with C-terminal c-Myc (Enl) or FLAG (Af9) tags. Mouse Cyclin T1, Dot1 (isoform a), Af10, NELF-E and DSIF p160 CTD (amino acids 748–1082) cDNA were amplified by RT–PCR from total RNA isolated from N2a cells (Cyclin T1) or testis tissue (Dot1, Af10, DSIF p160 CTD and NELF-E), and cloned into pCDNA3 in-frame with an N-terminal FLAG tag (DSIF p160 CTD and NELF-E). pCDNA3-Af4-wt/-mut, pCDNA3-Siah-1a and pM-Af4 (1–515) were previously described (14). Primer sequences and cycling conditions are available upon request.

**Cell culture and transfection**

HEK 293T, HeLa and N2a cells were cultured and transfected as previously described (14). Post-transfection, HEK293T were further treated for 1 or 7 h in the presence or absence of DRB or MG132 (Sigma) at 10 or 100 μM, respectively.

**RT–PCR analysis**

Total RNA was isolated using Tri-Reagent (Sigma) and reverse-transcribed using oligo (dT)12–18 primer (Invitrogen) and Expand Reverse Transcriptase (Roche) according to the manufacturer’s instructions. Primer sequences and PCR cycling conditions are available upon request.

**(Co)-immunoprecipitation and western blotting**

(Co)-Immunoprecipitation, BCA protein quantification, SDS–PAGE, western blotting and ECL detection were performed as previously described (14). Tissues were homogenized in co-immunoprecipitation buffer and analyzed as above.

**Immunohistochemistry**

Mice were transcardially perfused with 4% paraformaldehyde and brains were dissected and post-fixed overnight before embedding in paraplast wax. Sections of 10 μM were cut and mounted on lysine-coated slides. Samples were de-paraffinized with xylene and re-hydrated before antigen retrieval by boiling in 10 mM sodium citrate pH 6.0 for 20 min. Slides were blocked for 1 h in PBS containing 5% BSA and 0.5% Tween-20, and incubated overnight at room temperature with primary antibodies diluted according to the manufacturer’s instructions; the Af4 antiserum was used at a dilution of 1:750. After washes in PBS, sections were processed using the Vectastain ABC Elite (Vectorlabs) kit and appropriate secondary antibodies. Slides were stained using the DAB substrate kit (Vectorlabs), de-hydrated and mounted in Histomount (RA Lamb). Slides were observed under a phase contrast microscope (Leica), and images were captured using the Axiovision software (Axiocam).

**In vitro binding assay**

HA-Af4, Cyclin T1 and Cdk9 were purified from over-expressing HEK293T cells by immunoprecipitation as above, in the presence of 500 mM NaCl. Cdk9 and Cyclin T1 were eluted from the protein G matrix using the ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce) and dialyzed overnight in binding buffer (50 mM Tris pH 7.5, 150 mM NaCl). HA-Af4-bound protein G matrix was equilibrated in binding buffer and incubated for 30 min at RT with dialyzed Cdk9 or Cyclin T1. After extensive washing, HA-Af4-bound material was eluted from the matrix in SDS sample buffer, resolved by SDS–PAGE and analyzed by western blot using the appropriate antibodies.

**In vitro kinase assays**

For CTD kinase assays, 1 μg of Pol II CTD (Calbiochem) was incubated with 250 ng of P-TEFb in the presence or absence of HA-Af4 (purified as described above) and 10 μM of DRB for 1 h at 30°C in kinase buffer (50 mM Tris–HCl pH 7.5, 2 mM DTT, 5 mM MgCl2) supplemented with 1 mM ATP. Reactions were terminated by the addition of SDS sample buffer, resolved by SDS–PAGE and analyzed by western blot using the anti-phospho-Rpb1 CTD antibody. DSIF p160, NELF-E, Enl and Af9 kinase assays were performed as above in the presence of 2 μCi of [γ-32P] ATP (Amersham Biosciences), and analyzed by autoradiography. Recombinant FLAG-tagged DSIF p160 CTD and NELF-E, and Myc-Enl and FLAG-Af9 proteins were purified from over-expressing HEK293T cells following the same procedure as for HA-Af4.

**CAT ELISA transactivation assay**

The assay was performed in HeLa cells as previously described (14).

**Luciferase assay**

N2a cells were transfected with pGL2 control plasmid (Promega) and various pCDNA3 constructs for 36 h. Luciferase activity was measured using Dual-Glo luciferase assay system according to the manufacturer’s instructions (Promega).

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**Conflict of Interest statement.** None declared.
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