Human Polymerase-Associated Factor complex (PAFc) connects the Super Elongation Complex (SEC) to RNA polymerase II on chromatin

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The Super Elongation Complex (SEC), containing transcription elongation activators/coactivators P-TEFb, ELL2, AFF4/1, ENL, and AF9, is recruited by HIV-1 Tat and mixed lineage leukemia (MLL) proteins to activate the expression of HIV-1 and MLL-target genes, respectively. In the absence of Tat and MLL, however, it is unclear how SEC is targeted to RNA polymerase (Pol) II to stimulate elongation in general. Furthermore, although ENL and AF9 can bind the H3K79 methyltransferase Dot1L, it is unclear whether these bindings are required for SEC-mediated transcription. Here, we show that the homologous ENL and AF9 exist in separate SECs with similar but nonidentical functions. ENL/AF9 contacts the scaffolding protein AFF4 that uses separate domains to recruit different subunits into SEC. ENL/AF9 also exists outside SEC when bound to Dot1L, which is found to inhibit SEC function. The YEATS domain of ENL/AFF9 targets SEC to Pol II on chromatin through contacting the human Polymerase-Associated Factor complex (PAFc) complex. This finding explains the YEATS domain’s dispensability for leukemogenesis when ENL/AF9 is translocated to MLL, whose interactions with PAFc and DNA likely substitute for the PAFc/chromatin-targeting function of the YEATS domain.

Accumulating evidence has implicated the elongation stage of RNA polymerase II (Pol II) transcription as a major rate-limiting step for the expression of a large number of metazoan genes, especially those that control cell growth, renewal, and differentiation (1–3). During elongation, the processivity of Pol II is regulated by a set of transcription factors, which had been thought to exist as separate entities and impact on the Pol II elongation complex independently of one another. However, recent data from us and others indicate that at least two well-defined transcription elongation factors of different classes reside in a single multisubunit complex termed SEC [super elongation complex, (4)] to cooperatively activate transcription (4–6).

The first elongation factor found in SEC is human positive transcription elongation factor b (P-TEFb). Consisting of CDK9 (Cyclin-dependent Kinase 9) and cyclin T1 (CycT1), P-TEFb functions by phosphorylating the C-terminal domain (CTD) of the largest subunit of Pol II and negative elongation factors DSIF and NELF. These events antagonize the actions of the negative factors, release Pol II from promoter-proximal pausing, and trigger the production of full-length mRNA transcripts (7, 8). The second elongation factor in SEC is ELL2, which promotes elongation by keeping the 3′ OH of nascent mRNA in alignment with the catalytic site to prevent Pol II backtracking (9). Besides P-TEFb and ELL2, SEC also contains transcription factors/coactivators ENL, AF9, AFF4 (AF5q31), AFF1 (AF4), and probably others (4–6). Among these, AFF4 is known to mediate the interaction between ELL2 and P-TEFb and maintain the integrity of SEC (5). While AFF1 has been shown to interact with AFF4 (10), it remains to be seen whether the interaction between these two homologous proteins can exist in a single SEC complex.

The biological importance of SEC has been highlighted by recent discoveries that it is targeted by at least two sequence-specific transcriptional activators that play important roles in human diseases. First, the HIV-1 Tat protein is shown to interact with and recruit SEC to the HIV-1 LTR (Long Terminal Repeat) to stimulate viral transcription in a SEC-dependent manner (5, 6). In addition, Tat also promotes SEC formation, which in turn stabilizes ELL2, an otherwise short-lived protein rapidly degraded by the proteasome (5).

Besides HIV/AIDS, SEC is also found to be important for generating certain types of acute leukemias that involve chromosomal translocations of the mixed lineage leukemia (MLL) gene (4, 10, 11). At least three components of SEC, namely AFF4, ENL, and AF9, are known as fusion partners of MLL (12). When fused to the DNA-binding domain of MLL, these proteins deliver SEC and its powerful elongation stimulatory activity to the MLL-target genes to promote leukemic transformation (4, 10, 11).

Despite the demonstrations that SEC exists in cells under conditions that are free of Tat and MLL-translocations and is essential for metazoan transcriptional elongation in general (4–6, 10), little is known about how this complex is targeted to Pol II on a chromatin template in the absence of sequence-specific recruitment factors like Tat and MLL. Furthermore, compared to the other subunits of SEC, the contributions of ENL and AF9 to SEC function have not been well characterized. So far, the only clue to how these two proteins may be involved in gene expression control arises from their reported interactions with Dot1L (13, 14), a methyltransferase responsible for histone H3 lysine79 (H3K79) methylation (15, 16). As this modification is often found in actively transcribed regions (17, 18), the associations of ENL/AF9 with P-TEFb and Dot1L are believed to recruit the latter two into a large complex for efficient activation of transcription (12, 19), an intriguing proposition that is yet to be proven. Besides the uncertainty about the Dot1L-ENL/AF9 interaction, it is also unclear whether the evolutionarily conserved YEATS domain located in the N-terminal regions of ENL and AF9, which are often missing in the MLL-ENL/AF9 fusions and unimportant for leukemogenesis (20), is required for SEC function.

Here, presenting answers to these questions, we show that the highly homologous ENL and AF9 exist in separate SEC complexes that display similar but nonidentical functions. Within a


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SEC, ENL/AF9 is connected to the rest of the complex through the scaffolding protein AFF4, which uses separate domains to bind to different SEC subunits. Our data further show that Dot1L does not reside in or associate with SEC. Rather, Dot1L competes with AFF4 for binding to ENL/AF9 and inhibits SEC function. Finally, in the absence of sequence-specific recruitment factors, the YEATS domain of ENL/AF9 targets SEC to chromatin through contacting the human Polymerase-Associated Factor complex (PAFc) transcription elongation complex (21, 22), and through PAFc, the elongating Pol II. This observation explains why the N-terminal regions of ENL and AF9 are dispensable for leukemogenesis when fused to MLL, whose abilities to bind PAFc (23) and DNA likely bypass the requirement for the PAFc/chromatin-targeting function of the YEATS domain.

### Results

**ENL and AF9 Interact with P-TEFb Through the Scaffolding Protein AFF4s.** In light of our previous demonstration that AFF4 bridges the ELL2-P-TEFb interaction in SEC (5), we asked whether AFF4 plays a similar role in mediating the interactions of ENL and AF9 with P-TEFb. To address this question, short hairpin (sh)RNA-mediated depletion of AFF4 was performed in HeLa cells to assess its impact on the associations of ENL and AF9 with P-TEFb. Although the depletion did not affect the total levels of ENL and AF9 in nuclear extracts [nuclear extracts (NE); Fig. 1A, left box], depletion of AFF4 significantly reduced the levels of ENL and AF9 bound to the immunoprecipitated CDK9 (right box). Next, in vitro binding reactions were performed to test whether AFF4 could directly bridge the interaction between purified P-TEFb and ENL/AF9 in the absence of other SEC components. All the proteins added to the reactions were highly purified from transfected HeLa cells under stringent conditions (1.0 M KCl plus 0.5% NP-40) and confirmed by SDS-PAGE followed by silver-staining to be free of any contaminating proteins [Fig. S1; (5)]. As indicated in Fig. 1B, purified ENL and AF9 did not interact with immobilized CycT1-HA/CDK9 unless AFF4 was also present in the same reactions. Together, these in vitro and in vivo binding data highlight the important role for AFF4 to serve as a molecular scaffold to mediate the interactions of P-TEFb with not only ELL2 but also ENL and AF9.

ENL and AF9 Directly Interact with AFF4 Through Their C-Terminal Regions. Because AFF4 was shown to interconnect P-TEFb and ENL/AF9 in the absence of other SEC components, we reasoned that it must be able to make direct and simultaneous contacts with P-TEFb and ENL/AF9. In fact, a direct binding between AFF4 and P-TEFb has been demonstrated previously (5). To show that ENL and AF9 can also directly contact AFF4, in vitro binding reactions employing highly purified proteins (Fig. S1) were conducted. While wild-type (WT) ENL and AF9 were able to bind to immobilized HA-AFF4 directly, the C-terminally truncated ENL (1–431) and AF9 (1–480) were not (Fig. 1C). The requirement for the C-terminal regions of ENL and AF9 for binding to AFF4, which in turn allowed the formation of a complete SEC complex, was also confirmed in vivo in transfected cells (Fig. 1D and E).

Separate Regions of AFF4 Are Used to Interact with Different Subunits of SEC. Given the demonstrations that the scaffolding protein AFF4 can directly and simultaneously contact each and every subunit of SEC (Fig. 2, (5)), we would like to map its regions that are involved in these interactions. A series of Flag-tagged AFF4 deletion mutants were tested for their ability to coprecipitate the other components of SEC. When the levels of WT and mutant AFF4 in the antiFlag immunoprecipitates (IP) were normalized to a similar level, the mutant missing the first 300 residues, ∆1-300, was found to interact with ELL2, ENL, and AF9 normally but not CDK9 (Fig. 2, lane 3). In contrast, a short AFF4 truncation mutant containing just the first 300 amino acids (1–300) was able to efficiently pull-down CDK9 but not any other components of SEC (lane 6). Thus, the first 300 residues of AFF4 contain an independent domain that is necessary and sufficient for P-TEFb-binding. Similarly, the region between aa 301 and 600 in AFF4 appears to contain an ELL2-binding domain. This notion is indicated by the demonstration that the deletion mutant ∆1-600 failed to interact with ELL2 and P-TEFb, but still retained WT ability to bind to ENL and AF9 (Fig. 2, lane 4). On the other hand, a 300 residue-long segment of AFF4 encompassing positions 301 to 600 (301–600) displayed reduced but clearly above-the-background level of binding to ELL2 but not any other components of SEC (lane 7). Finally, the region between amino acids 601 and 900 of AFF4 likely contains an independent binding domain for ELL2.

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**Fig. 1.** The scaffolding protein AFF4 directly binds to the C-terminal regions of ENL and AF9 to mediate their interactions with P-TEFb. (A) NE were prepared from HeLa cells either containing an empty vector or expressing the AFF4-specific shRNA (shAFF4) and subjected to immunoprecipitation (IP) with anti-CDK9 or an irrelevant rabbit IgG as a control. The isolated NE (left box) and immunoprecipitates (right box) were analyzed by Western blotting with the indicated antibodies. (B) The indicated highly purified proteins were incubated with immobilized CycT1-HA/CDK9 in vitro and the bound proteins were eluted and analyzed by Western blotting (right). Five percent of the input proteins were also examined by anti-Flag Western blotting (left). (C) In vitro binding reactions were performed by incubating highly purified WT or C-terminally truncated ENL-F or AF9-F (schematic diagram on the right) with immobilized HA-AFF4. The bound proteins and 10% of the soluble input proteins were analyzed by Western blotting with the indicated antibodies. (D) WT or mutant ENL and AF9, all Flag-tagged, were expressed in transfected HeLa cells. Anti-Flag immunoprecipitates were examined by Western blotting for the ENL/AF9-associated factors.

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and AF9, as the fragment encompassing this region interacted efficiently with these two proteins but not any other subunits of SEC (lane 8) and the deletion mutant lacking the entire N-terminal 900 amino acids (Δ1-900) failed to associate with any component of SEC (lane 5). Together, these data indicate that AFF4 uses separate domains to interact with different subunits of SEC and serves as a platform to nucleate the assembly of SEC.

**ENL and AF9 Exist in Separate SEC Complexes.** ENL and AF9 are highly homologous proteins, with their N-terminal YEATS domains and C-terminal coiled-coil domains showing a particularly high degree of identity (82%). Given this homology and the above demonstration that both proteins bound to the same region (aa 601–900) of AFF4 (Fig. 2), we asked whether they exist simultaneously in a single SEC complex. To address this question, anti-Flag immunoprecipitations were performed in extracts of cells coexpressing HA-tagged AF9 and Flag-tagged ENL or AFF4. While AF9-HA coprecipitated with F-AFF4 and CDK9 as expected, it did not coprecipitate with ENL-F. The reciprocal co-IP experiment employing anti-HA beads also failed to detect the interaction between AF9-HA and ENL-F. These data suggest that AF9 and ENL do not exist in the same complex, although each can establish independent interactions with other SEC subunits.

To obtain further evidence in support of this notion, we performed immunodepletion to remove all ENL proteins from HeLa NE (Fig. 3B). This procedure caused the codepletion of more than 75% of AFF4, ~30% of CDK9, but very few AF9 and the internal control protein, α-Tubulin, from NE. This result is consistent with the idea that ENL and AF9 do not exist in the same SEC complex. Because a major fraction of nuclear CDK9 is normally sequestered in the 7SK snRNP (8), it is not surprising to see that more than half of CDK9 were still present in the ENL-depleted NE. However, the codepletion of more than 70% of AFF4 suggests that the AF9-containing SEC contains at most 30% of total AFF4 in NE, assuming that no AFF4 exists outside of the SEC complexes. Thus, the ENL-containing SEC appears to be the predominant form between the two SEC complexes.

**ENL and AF9 Compete for Binding to AFF4.** A likely reason for the failure of ENL and AF9 to coexist in the same SEC is revealed subsequently in an in vitro binding assay employing highly purified proteins (Fig. S1). The addition of increasing levels of WT AF9-F but not the C-terminally truncated AF9-F 1-480 that is defective for AFF4-binding (Fig. 1C), into binding reactions progressively reduced the amounts of F-AFF4 bound to the immuno-bobilized ENL-HA (Fig. 3C). Thus, the bindings of AF9 and ENL to AFF4, which occur through the same region (aa 601–900) of AFF4 (Fig. 2), were mutually exclusive, which explains their failure to exist in the same SEC complex.

**Loss of ENL Is Compensated by Increased AF9 Expression, but Not Vice Versa.** The existence of two versions of SEC with one containing AF9 and the other ENL makes it important to examine whether these two homologous proteins have similar or different functions. Toward this goal, stable shRNA-mediated depletion of either ENL or AF9 was performed and the effect on SEC formation was assessed by anti-CDK9 immunoprecipitation followed by Western blotting. Notably, the reduction of total and CDK9-bound ENL in HeLa cells by shENL triggered a marked increase in the amounts of AF9, but not AFF4 or ELL2, in both NE and anti-CDK9 IP (Fig. 3D). In contrast, shRNA depletion of AF9 did not produce any significant change in ENL expression or sequestration into SEC (Fig. 3E). Thus, the loss of the ENL-containing SEC was compensated for the increased formation of the AF9-containing SEC, but not vice versa. This phenomenon is likely caused by the fact that the former complex is the predominant form between the two SECs, and the compensation serves to prevent a major reduction in the overall SEC level in the cell.

**AF9 and ENL Have Similar but Nonidentical Functions in Mediating SEC-Dependent Transcription.** In agreement with the above demonstration that AF9 increased expression to compensate for the loss of ENL but not vice versa, we consistently observed that shRNA depletion of ENL had a smaller effect on HIV-1 transcription, which is shown to depend on the SEC function (5, 6), than did the depletion of AF9. For example, using the HIV-1 LTR-driven luciferase expression as readout, shENL reduced basal and Tat-activated HIV LTR activity by 29% and 24%, respectively (Fig. 3F and G), whereas shAF9 decreased the two by 40% and 37%, respectively, when the knockdown efficiency was normalized between the two shRNAs. Despite this difference, the overall impact on the HIV-1 LTR by the individual depletion of AF9 or ENL was relatively minor. Only when both proteins were codepleted at the same time, a more drastic reduction in HIV-1 transcription (72% reduction for basal and 69% for Tat-activated transcription) was observed (Fig. 3F and G). The fact that the depletion of ENL or AF9 alone only partially suppressed SEC-dependent HIV-1 transcription whereas the depletion of both had a much more significant effect indicates that the two proteins have similar but not completely identical functions.

**Dot1L Is Not a Component of SEC.** The identification of ENL/AFF9 as a subunit of SEC raises the issue of whether the methyltransferase Dot1L, a well known partner of ENL and AF9 (15, 14), also exists and plays a key role in this complex. Dot1L is responsible for the methylation of H3K79 (15, 16), a modification that is often associated with actively transcribed genes (17, 18). Because of their overall positive influence on transcription and common connection through ENL/AFF9, Dot1L, and the key SEC subunits P-TEFb and ELL have been depicted in recent models as components of a larger complex for coordinated activation of transcription (11, 12, 19).
To determine whether Dot1L is associated with SEC, anti-Flag immunoprecipitations were performed in NE of HeLa cells expressing F-Dot1L or F-AFF4. While F-AFF4 readily co-purified with the SEC components ENL, AF9, and CDK9 as expected, F-Dot1L was only able to pull-down about the same amounts of ENL and AF9 but not CDK9 under identical conditions (Fig. 4A). This observation suggests that the interactions of Dot1L with ENL/AF9 likely occurred outside of SEC, a notion also proposed by Lin et al. (4, 10, 11) without showing the data.

It has previously been shown that HIV-1 Tat can bind to and promote the formation of SEC (5, 6). Consistent with this observation, transiently expressed Tat-HA precipitated SEC subunits CDK9 and AFF4 but not Dot1L, although a separate ENL-Dot1L interaction was easily detected in the same experiment (Fig. 4B).

ENL/AF9 Cannot Simultaneously Interact with AFF4 and Dot1L. The data in Fig. 1 show that ENL and AF9 use their C-terminal regions to contact AFF4. These same regions, however, are also responsible for direct interactions of ENL and AF9 with Dot1L (13, 14). To explain why Dot1L was not detected in SEC, we asked whether ENL/AF9 could bind to Dot1L and AFF4 at the same time. To answer this question, an in vitro binding/competition assay employing highly purified proteins (Fig. S1) was performed. In agreement with the data in Fig. 1, only WT ENL-F, but not the C-terminally truncated ENL-F (5%) 1-480, bound to the immobilized ENL-HA (Fig. 4C). This observation was also obtained with AF9 (Fig. 4D). A similar observation was also obtained with AF9 (Fig. 4D). Thus, Dot1L...
and AFF4 directly compete for binding to ENL and AF9, which explains why Dot1L was not detected in SEC. These results are consistent with the observations by Yokoyama et al. (10) that ENL coprecipitated with Dot1L and AFF4 when coexpressed in HEK293 cells and that Dot1L and AFF4 failed to coprecipitate under the same conditions.

**Dot1L Inhibits SEC-Dependent Transcription.** Even though Dot1L is not an integral component of SEC, we asked whether it might still be required for basal and Tat-activated HIV-1 transcription, which is shown to proceed in a SEC-dependent manner (5, 6). To this end, the siRNA-mediated silencing of Dot1L expression was performed, which was found to also significantly reduce the nuclear level of H3K79me2 but not AF9 as expected (Fig. 4F). Surprisingly, the loss of Dot1L and H3K79me2 not only failed to inhibit the HIV-1 LTR; it actually enhanced the luciferase production from a stably integrated HIV-1 LTR-driven luciferase reporter gene under both Tat(+) and (−) conditions (Fig. 4E). It is interesting to note not all genes depend on Dot1L for expression. The data above place the HIV-1 LTR among a group of actively transcribed genes that are known to be inhibited by Dot1L (24, 25).

**The YEATS Domains of ENL and AF9 Are Unimportant for SEC Formation but Essential for SEC-Dependent Transcription.** ENL and AF9 are two of the most frequent MLL fusion partners for inducing acute leukemias. When fused to MLL, their C-terminal regions are necessary and sufficient for oncogenic activities of the fusion products (10, 20). In contrast, their N-terminal regions that contain the highly conserved YEATS domain (26) are largely dispensable for leukemic transformation.

To determine whether the YEATS domains of AF9 and ENL play any role in SEC function, we compared WT AF9 and ENL and their mutants lacking the N-terminal YEATS domain for their abilities to promote luciferase gene expression driven by the HIV-1 LTR, whose activity requires a functional SEC (5, 6). Whereas WT AF9 and ENL activated the LTR by 9.3- and 5.4-folds, respectively, the deletion mutants produced only 1.3- and 2.1-fold increase, despite their stable accumulation in transfected cells (Fig. 5A). These results reveal a critical role for the YEATS
domains in mediating the SEC-dependent HIV-1 transcription. However, our subsequent co-IP experiments indicate that like their WT counterparts, the N-terminal deletion mutants of AF9 and ENL were fully capable of interacting with all the other components of SEC (Fig. 5 B and C). Thus, the YEATS domains contribute to the SEC function not through maintaining the integrity of the complex.

The YEATS Domains of ENL and AF9 Display Chromatin-Targeting Function. Given the observations that the YEATS domains are crucial for AF9/ENL to support the SEC-dependent HIV-1 transcription but dispensable for MLL-AF9/ENL to cause leukemia (10, 20), we wanted to know exactly how this domain contributes to SEC function. Because MLL confers the chromatin-targeting function to the MLL-AF9/ENL fusions, we asked whether the YEATS domain plays a similar role in promoting the interaction of SEC with chromatin in the absence of a sequence-specific recruitment factor like Tat or MLL. To this end, the chromatin immunoprecipitation (ChIP) assay was performed to test the associations of WT ENL and AF9 as well as their YEATS domain deletion mutants with a stably integrated HIV-1 LTR-luciferase reporter gene. While WT ENL and AF9 were detected readily on the chromatin template at the promoter, interior of the luciferase gene. While WT and N-terminally deleted AF9-F and ENL-F in transfected cells. (B and C). Hela cells were transfected with the indicated cDNA constructs. NE (left boxes) and anti-Flag immunoprecipitates isolated from NE (right boxes) were examined by Western blotting for the presence of the indicated proteins. A non-specific band is indicated by asterisks (*).

The YEATS Domain Directly Binds to the PAF1 Subunit of PAFc to Target SEC to Chromatin. What could be the functional target on chromatin that is used by the YEATS domain to deliver SEC to Pol II? Recently, an interaction of SEC with the PAFc, which is known to associate with Pol II during productive elongation (21, 27), has been discovered in cells expressing HIV-1 Tat (6). It is yet to be shown whether this interaction also occurs under Tat(−) conditions and what role it may play during SEC-dependent transcription. As the first step toward answering these questions, we asked whether the YEATS domain might be required for the SEC-PAFc interaction. Indeed, compared to WT ENL, the mutant missing the N-terminal YEATS domain (aa 113–559) interacted normally with CDK9 but not PAF1, the scaffolding subunit of the multi-component PAFc (Fig. 6C). In comparison, the ENL mutant lacking the C-terminal AFF4/P-TEFb-binding domain (aa 1–430) showed WT interaction with PAF1 but not CDK9.

Just like ENL, AF9 also depended on the YEATS domain to interact with PAF1 (Fig. 6D). The subsequent in vitro GST pull-down assay further reveals a direct physical interaction between recombiant PAF1 and the ENLYEATS domain that is fused to GST (GST-ENL-N; Fig. 6C). In contrast, GST-ENL-C, which retains the C-terminal AFF4/P-TEFb-binding region, failed to bind to PAF1.

PAFc Connects SEC to Pol II on Chromatin Templates. Given that the YEATS domain mediated the interaction of SEC with PAFc, which is known to associate with Pol II during elongation (21), we postulated that this domain must also play a key role in allowing SEC to ultimately reach its functional target Pol II. Indeed, co-IP experiments in Fig. 7A reveal that compared to WT ENL, the YEATS-deleted mutant (aa113–559) consistently showed decreased binding to RBP1, the largest subunit of Pol II. To ensure that the observed interactions with endogenous PAF1 and RBPI...
The error bars represent mean ± SD. The levels of WT and mutant AF9-F or ENL-F in NE were examined by anti-Flag Western blotting in the right boxes. (C and D). HeLa cells were transfected with the indicated ENL-F (C) or AF9-F-expressing constructs (D). NE (left boxes) and anti-Flag immunoprecipitates isolated from NE (right boxes) were examined by Western blotting for the presence of the indicated proteins. A nonspecific band in (D) is indicated by an asterisk (*). (E). The GST pull-down assay was performed with the indicated proteins present in the reactions. After extensive washing, the proteins bound to the GST beads were detected by silver staining. GST-ENL-N and GST-ENL-C contain amino acids 1-154 and 433-559 of ENL, respectively.

by transfected WT ENL-F and AF9-F are not a result of transient overexpression of the latter two proteins, we expressed specific shRNAs to enable simultaneous knockdown of both ENL and AF9, which were shown to exist in two separate SECs and display similar functions (Fig. 3). Confirming the dependence on the YEATS-containing ENL/AF9 for the interactions of endogenous

Fig. 6. ENL/AF9 YEATS domain interacts directly with PAF1 to target SEC to a chromatin template. (A and B). ChIP with the anti-Flag antibody was performed in cells containing an integrated HIV-1 LTR-luciferase reporter gene and transiently expressing WT or N-terminally deleted AF9-F (A) or ENL-F (B). Three regions corresponding to the promoter, interior, and 3' UTR of the integrated reporter gene were qPCR-amplified from the precipitated and purified DNA and shown as percentages of the input chromatin. The error bars represent mean ± SD. The levels of WT and mutant AF9-F or ENL-F in NE were examined by anti-Flag Western blotting in the right boxes. (C and D). HeLa cells were transfected with the indicated ENL-F (C) or AF9-F-expressing constructs (D). NE (left boxes) and anti-Flag immunoprecipitates isolated from NE (right boxes) were examined by Western blotting for the presence of the indicated proteins. A nonspecific band in (D) is indicated by an asterisk (*). (E). The GST pull-down assay was performed with the indicated proteins present in the reactions. After extensive washing, the proteins bound to the GST beads were detected by silver staining. GST-ENL-N and GST-ENL-C contain amino acids 1-154 and 433-559 of ENL, respectively.

Fig. 7. PAFc connects SEC to Pol II. (A). HeLa cells were transfected with the indicated ENL-F-expressing constructs. NE (left boxes) and anti-Flag immunoprecipitates isolated from NE (right boxes) were examined by Western blotting for the presence of the indicated proteins. (B and C). NE from HeLa cells either containing an empty vector or expressing the indicated shRNAs (B) or from the inducible shPAF1-expressing cells treated with (+) or without (−) doxycycline (Dox) to induce shPAF1 expression (C) were subjected to IP with the indicated antibodies. The isolated NE (left) and immunoprecipitates (right) were analyzed by Western blotting with the indicated antibodies. (D). The ChIP assay was performed in HeLa cells with either the anti-CDK9 or an irrelevant control antibody. Interior regions of the c-Myc and HEXIM1 gene were amplified by qPCR from the precipitated and purified DNA and shown as percentages of the input chromatin. The error bars represent mean ± SD. (E). A model showing the recruitment of the SEC complex, which contains either ENL or AF9 and is assembled around the scaffolding protein AF4, to the elongating Pol II through the interaction of the ENL/AF9 YEATS domain with the PAF1 subunit of PAFc. This configuration allows SEC to use its P-TEFb and ELL2 functional modules to exert a multitude of effects that include the phosphorylation of the Pol II CTD and elongation factors DSIF and NELF (the latter is released upon phosphorylation) by CDK9 and the suppression of Pol II pausing by ELL2. These events synergistically activate productive elongation and likely also transcription-coupled mRNA 3′ processing.
SEC with PAFs and Pol II, the combination of shENL and shAF9 markedly decreased the amounts of PAF1 and RPBI associated with the immunoprecipitated CDK9 (Fig. 7B).

Theoretically speaking, the above-described YEATS domain-dependent interaction between SEC and Pol II could proceed independently of PAFc. To prove that PAFc indeed acts as a bridge to interconnect SEC and Pol II, we performed shRNA-mediated depletion of PAF1 under inducible conditions. Upon the induction of PAF1 depletion by doxycycline, which activated shPAF1 expression, a significant reduction in the amount of RPBI bound to the immunoprecipitated CDK9-F, a key SEC subunit, was observed (Fig. 7C).

Given the important role for PAFc in mediating the interaction of SEC with elongating Pol II, we predicted that PAFc must also be required to target SEC to chromatin templates that encompass endogenous genes. Indeed, siRNA-mediated PAF1 knockdown in HeLa cells was found to markedly reduce the associations of the SEC subunit CDK9 with two endogenous gene loci c-Myc and H3XIM1 (Fig. 7D), which have been shown to depend on P-TEFb for expression (28, 29). Very similar observations were also obtained in 293T cells. Taken together, the data above support a model (Fig. 7E) that the YEATS domain of ENL/AF9 functions as a bridge to connect SEC to PAFc, and through PAFc, the elongating Pol II on a chromatin template. Once positioned next to Pol II, SEC applies its two functional modules, P-TEFb and gating Pol II on a chromatin template. Once positioned next to SEC. Rather, it competes with AFF4 for binding to ENL/AF9, suggesting that the Dot1L-ENL/AF9 interactions occur outside of the SEC complex, a conclusion that was also reached under different experimental conditions (10). Consistent with this conclusion, it was recently shown that the MLL-ENL fusion delivers Dot1L and SEC as two separate entities to the MLL-target loci. However, contradicting with the general presumption of the field, it is the recruitment of SEC that contributes predominantly to MLL-dependent leukemogenesis (10).

The methylation of H3K79, including mono-, di- and trimethylation, is likely carried out exclusively by Dot1L (16). So far, these modifications marks have displayed a complex relationship with gene transcription. For example, genome-wide analyses in Drosophila indicate that hypermethylated H3K79 is frequently enriched within actively transcribed genes, whereas hypomethylated H3K79 is generally associated with inactive genes (18). A subsequent high-resolution profiling of histone methylations in the human genome has painted a more detailed picture by showing that while monomethylation of H3K79 is linked to gene activation, trimethylation is associated with repression (24). In an attempt to examine the relationship between H3K79 methylation and transcriptional control at a single gene locus, it was found that H3K79 dimethylation marks developmental activation of the β-globin gene but is reduced upon LCR-mediated high-level transcription (25). Taken together, these studies send a clear message that H3K79 methylation and its responsible enzyme Dot1L play complicated and sometimes conflicting roles in controlling gene expression. Depending on the specific genes and conditions involved, H3K79 methylation and Dot1L can exert either a positive or negative influence on transcription. The inhibitory effect of Dot1L on HIV-1 transcription observed in the current study provides yet another example supporting the notion that Dot1L and H3K79 methylation are not always associated with transcriptional activation.

The final important finding of the current study is about the highly conserved YEATS domain in ENL and AF9. Many YEATS domain-containing proteins are components of histone-modifying and transcription complexes (26). However, the function of this domain itself remains poorly understood thus far. Prior to the current study, the only clue suggesting how it might contribute to the activity of SEC comes from the observation that the YEATS domain of ENL interacts with histones H3 and H1 in vitro (31). These interactions, which are yet to be confirmed in vivo, could in principle play a role in attracting SEC to a chromatin template. However, because H3 and H1 are not specifically associated with active transcription, their interactions with ENL/AF9 are not expected to recruit SEC to only the actively transcribed genes, let alone to keep SEC continuously engaged in comigrating with its functional target, Pol II, during elongation.

These tasks, while challenging for histones H3 and H1, would be fairly straightforward and natural for the multisubunit and multifunctional PAFc to accomplish. The reason is because PAFc is well known for its association with the elongating Pol II (21, 22, 32), contribution to transcriptional elongation on chromatin templates (21) and participation in transcription-coupled mRNA 3′ processing (33). Furthermore, PAFc also interacts directly with SEC, although the physiological significance of this interaction was unknown at the time (6). These functions make PAFc an ideal candidate for the YEATS domain of ENL/AF9 to bind and deliver SEC to Pol II on a chromatin template. Indeed, data presented here completely agree with this notion and indicate that the YEATS domain directly binds to the PAF1 subunit, which is a scaffolding molecule to reinforce the binary interactions between other subunits of PAFc (21). This interaction is shown to target SEC to Pol II and chromatin and allow SEC to stimulate productive elongation and likely also transcription-coupled mRNA polyadenylation. This latter role of SEC is supported by the demonstrations that the SEC component P-TEFb plays a key role in 3′ processing and that the distribution of PAFc is concentrated toward the 3′ end of genes (34–37).
It is interesting to note that SII/TFIIS, another well known transcription elongation factor that acts by inducing transcript cleavage in arrested elongation complexes and permitting paused Pol II to proceed downstream, was recently shown to cooperate with PAFc to bind to Pol II and stimulate elongation (21). It will be interesting to test whether the interactions of PAFc with SII and SEC occur simultaneously or in an exclusive manner, which will determine whether different elongation activities as represented by SEC and SII can work on the same polymerase enzyme to achieve synergistic activation.

The discovery of the chromatin/PAFc-targeting function of the YEATS domain has also helped clarify a long-standing confusion stemming from the observations that this domain confers autonomous transactivation of the SV40 minimal promoter (31) and contributes to the SEC function (Fig. 5A) but is nevertheless dispensable for oncogenic transformation in the context of the MLL-ENL/AF9 translocations (10, 20). It is interesting to note that the MLL portion of the fusion proteins contains multiple DNA-binding structures that enable both sequence-specific and -nonspecific bindings to the target loci (20) and also the CxxC-RD2 domain for interacting with PAFc (23). It is highly likely that these activities of MLL, which are known to be essential for transformation, can effectively substitute for the PAFc/chromatin-targeting function of the YEATS domain. Besides ENL and AF9, 132 additional proteins in 59 different eukaryotes are also known to possess the YEATS domain (26). Future studies will shed light on whether the ability of this domain to target chromatin, PAFc, and Pol II is evolutionarily conserved and how it may contribute to the biological functions of diverse YEATS domain-containing proteins.

**Experimental Procedures**

**Antibodies.** The anti-ENL (A302-267A), -AF9 (A300-595A) and -ELL2 (A302-505A-1) antibodies were purchased from Bethyl Laboratories, Inc.. The anti-Dot1L (ab72454) and anti-H3 di-ELL2 (A302-505A-1) antibodies were purchased from Abcam. The antibodies against CDK9 and AFF4 have been described previously (5).

**Chromatin Immunoprecipitation (ChIP) Assay.** The ChIP assay was carried out essentially as described (38) with minor modifications. After formaldehyde fixation, HeLa cells (2 × 10^5) or a HeLa-based cell line containing a stably integrated HIV-1 LTR-Luciferase reporter gene and transfected with the indicated ENL/AF9-expressing constructs were incubated in lysis buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl and 1% NP-40) on ice for 10 min and the nuclei were collected and resuspended in sonication buffer (15 mM Tris-Hcl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl). For RNAi-coupled ChIP analyses, HeLa cells were treated for 48 h with specific siRNA duplexes (Dharmacon) using INTERFERin™ (Polyplus) and then subjected to ChIP analyses as mentioned above. Primers used to amplify the HEXIM1 gene are: forward 5′-TAACAC-GACGCGATCTCTATATAGA-3′ and reverse 5′-TGAGCTCTCT-GTGTGCTATGTCTACT-3′. For the c-Myc gene, the forward primer is: 5′-ACTCTGTCGACGCCATTTCTACT-3′ and the reverse primer is: 5′-GCACGACTGTAATCTTTCCAGA-3′. The nucleotide sequences of the PCR primers used to examine the occupancy of ENL/AF9 at the HIV-1 promoter, the interior of the luciferase coding sequence and the 3′ untranslated region (3′ UTR) were described previously (5).

**Generation of Inducible PAF1 Knockdown Cells.** The DNA oligonucleotide encoding the PAF1-specific shRNA (shPAF1; 5′-GAT-CAAGTGCGGAATGAAGAAATCATTGCCTACGTCACTCC-3′) was cloned into the pSuperior.retro.neo + GFP construct (OligoEngine), which was introduced by retroviral infection into the T-REX-TM293 (Invitrogen)-based cell line stably expressing CDK9-F as described previously (5). The procedures for the production of recombinant retroviruses, infection of cells, and generation of neomycin-resistant colonies have been described previously (5). Single colonies were picked and screened for inducible knockdown of endogenous PAF1 upon the treatment with doxycycline (1 µg/mL) for 2 d.

**Generation of ENL and AF9 Knockdown Cells.** The procedure for generating a HeLa-based cell line in which the expressions of ENL and AF9 were silenced simultaneously has been described previously (5). The shRNA sequences used in the current procedure are:

- **ENL-ctrl;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′
- **ENL-sh1;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′
- **ENL-sh2;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′
- **AF9-ctrl;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′
- **AF9-sh10;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′
- **AF9-sh30;** 5′GATCAATCGATGCGGATGTCAGGTTCCTGTC-3′

**siRNA-Mediated Knockdown of Dot1L Expression.** HeLa cells containing an integrated LTR-luciferase reporter construct were transfected twice with Dot1L-specific or control scrambled siRNA (ctl.). After six days of transfection cells were transduced with a retrovirus encoding for Tat or empty vector. siRNA sequences used: Ctrl.: 5′-auguauuccgguaauagtt-3′; Dot1L: 5′-uguauuggcgccauguuggt-3′.
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Expression and Purification of Recombinant PAF1, GST-ENL-N, and GST-ENL-C. Full-length PAF1 cDNA was inserted into the pET28b expression vector (Invitrogen) containing an N-terminal (His)$_6$ tag and thrombin cleavage site. ENL N-terminal (1–154) and C-terminal (434–560) fragments were inserted into pGEX-6P-3 vectors (GE Healthcare) containing an N-terminal GST tag and PreScission Protease cleavage site. Tagged proteins were expressed in *Escherichia coli* BL21 CodonPlus-(DE3)-RIL cells (Stratagene) through growth in TB media at 37 °C until OD 0.5, followed by induction with 100 uM IPTG for 16 h at 16 °C. Cells were harvested by centrifugation, and pellets were stored at −80 °C.

GST-ENL-N and GST-ENL-C bacterial pellets were resuspended in GST-Lysis Buffer (500 mM NaCl, 20 mM Hepes pH 8.0, 0.5 mM TCEP, 10% glycerol), and His-PAF1 pellets were resuspended in NiA-Lysis Buffer (500 mM NaCl, 20 mM Hepes pH 8.5, 0.5 mM TCEP, 10% glycerol, 25 mM imidazole) in the presence of protease inhibitors. Cell suspensions were lysed by sonication, followed by centrifugation at 17,000 × g for 60 min. Clarified lysates were applied to the GSTrap FF column or HisTrap FF columns (GE Healthcare) for affinity purification on the AKTA Explorer FPLC through gradient elution using GST Elution Buffer (500 mM NaCl, 20 mM Hepes pH 8.0, 0.5 mM TCEP, 10% glycerol, 25 mM glutathione) or Ni Elution Buffer (500 mM NaCl, 20 mM Hepes pH 8.0, 0.5 mM TCEP, 10% glycerol, 350 mM imidazole). Fractions containing His-PAF1 were pooled and incubated with thrombin (1:500) for 12 h at 4 °C for tag cleavage. The cleaved His-tag was removed using a second HisTrap FF column.

PAF1 and GST-ENL proteins were further purified by size exclusion chromatography using the Superdex 200 16/60 (GE Healthcare) in S200 Buffer (150 mM NaCl, 20 mM Hepes pH 8.0, 1 mM TCEP). Elution volumes were checked against standards to confirm the expected size of the proteins, based on sequence, SDS-PAGE, and Western blotting results. Full-length PAF1 eluted at a calculated size of 78.6 kD (monomer). GST-ENL-N eluted at 134.1 kD, and GST-ENL-C eluted at 122.5 kD due to GST-tag size and dimerization. Fractions containing PAF1, GST-ENL-N, and GST-ENL-C were pooled and concentrated to 100 μg/mL for pull-down assays.

**Fig. S1.** Purification of epitope-tagged AFF4, Dot1L, wild-type and C-terminally truncated ENL and AF9 from transfected HeLa cells under highly stringent conditions involving high salt (1.0 M KCl) and detergent (0.5% NP-40) was conducted. The proteins were examined by SDS-PAGE followed by silver-staining to show that they are free of any associated proteins. The minor, faster migrating doublet in the AFF4 lane denoted by an asterisk (*) is confirmed as truncated AFF4.