The transition from transcription initiation to elongation is a key regulatory step in gene expression, which requires RNA polymerase II (pol II) to escape promoter proximal pausing on chromatin. Although elongation factors promote pause release leading to transcription elongation, the role of epigenetic modifications during this critical transition step is poorly understood. Two histone marks on histone H3, lysine 4 trimethylation (H3K4me3) and lysine 9 acetylation (H3K9ac), co-localize on active gene promoters and are associated with active transcription. H3K4me3 can promote transcription initiation, yet the functional role of H3K9ac is much less understood. We hypothesized that H3K9ac may function downstream of transcription initiation by recruiting proteins important for the next step of transcription. Here, we describe a functional role for H3K9ac in promoting pol II pause release by directly recruiting the super elongation complex (SEC) to chromatin. H3K9ac serves as a substrate for direct binding of the SEC, as does acetylation of histone H4 lysine 5 to a lesser extent. Furthermore, lysine 9 on histone H3 is necessary for maximal pol II pause release through SEC action, and loss of H3K9ac increases the pol II pausing index on a subset of genes in HeLa cells. At select gene promoters, H3K9ac loss or SEC depletion reduces gene expression and increases paused pol II occupancy. We therefore propose that an ordered histone code can promote progression through the transcription cycle, providing new mechanistic insight indicating that SEC recruitment to certain acetylated histones on a subset of genes stimulates the subsequent release of paused pol II needed for transcription elongation.

Transcription mediated by RNA polymerase II (pol II) occurs in a series of ordered steps in the transcription cycle, ranging from initiation to elongation to termination, with each step requiring the coordination of a vast array of proteins to a target gene promoter in a specific sequential manner (1). The transitions between these steps are highly regulated and often targeted in disease (2, 3), making these processes vital to study for understanding cell biology and pathology. pol II promoter proximal pausing is thought to be a key regulatory step that occurs on the majority of mammalian genes (2, 4). Typically, just downstream of the transcription start site (TSS), pol II will pause, and elongation factors are then required to release pol II for subsequent productive elongation. Although many protein complexes have been identified to facilitate pol II pause release, the function of epigenetic modifications in the local chromatin environment is unknown and may have key regulatory roles in this process. Different steps of transcription are associated with different post-translational modifications on histones, yet the extent to which specific epigenetic modifications drive functional processes in transcription remains incompletely understood. The N-terminal end of histone tails is subject to extensive modifications, including the methylation and acetylation of lysine residues, which can affect chromatin structure and function to recruit effector proteins, or “readers,” through specific domains (5). Furthermore, the combination and the order of these histone marks can influence specific downstream functions in a cell (6).

We previously discovered that nucleosomes containing only trimethylation on histone 3 at lysine 4 (H3K4me3) promote acetylation selectively on the neighboring residue lysine 9 (H3K9ac) in a cell-free histone acetyltransferase (HAT) assay (7). This interplay between H3K4me3 and H3K9ac occurs through the binding action of the HAT complex subunit SAGA complex-associated factor 29 (SGF29) to H3K4me3, which is necessary for maximal acetylation of H3K9 (8, 9) and is medi-
determined by the HATs general control non-derepressible 5 (GCN5) and p300/CBP-associated factor (PCAF) (7, 10). GCN5 and PCAF function to catalyze acetylation as subunits in two distinct HAT complexes, SAGA (Spt-Ada-Gcn5 acetyltransferase) and ATAC (Ada2a-containing) (11–15). However, the specific mechanistic role that H3K9ac may have in transcription is unclear. The presence of histone acetylation is associated with gene expression (16–19) and reduces DNA-histone binding by neutralizing the positive charge of lysine (20). Despite this correlative data, the causal role of specific histone acetylation marks during transcription and the breadth of effector proteins remain to be determined. H3K4me3 is a well-studied histone mark that stimulates transcription (7, 21, 22), and its genomic occupancy positively correlates with histone acetylation across cell types (16). H3K4me3 localizes at actively transcribing gene promoters (16, 17) and can recruit myriad co-regulators and transcriptional machinery (23, 24). In addition to HATs, H3K4me3 also serves as a substrate for TATA-box-binding protein-associated factor 3 (TAF3) (22, 25). Thus, H3K4me3 is a critical pol II transcription initiation mark as it aids in pre-initiation complex formation by TFIIID recruitment.

The super elongation complex (SEC) is a multisubunit complex that is important in the transition from transcription initiation to elongation, as it functions to release pol II from a paused state on active genes (26). The enzymatic subunit, positive transcription elongation factor b (P-TEFB), mediates phosphorylation of both negative elongation factors and serine 2 of the C-terminal domain of the largest subunit of pol II, resulting in productive transcription elongation (27–30). Additional complex subunits of the SEC include the AF4/FMR2 family members 1 or 4 (AFF1 or 4), eleven-nineteen Lys-rich leukemia (ELL) proteins ELL1, ELL2, or ELL3, and the YEATS domain-containing proteins eleven-nineteen leukemia (ENL) and ALL1 fused gene from chromosome 9 (AF9) (26).

We hypothesized that H3K4me3 promotes select histone acetylation for advancement to the next stage in the pol II transcription cycle. Here, we report that both H3K9ac and H4K5ac are substrates for direct binding of the SEC through the YEATS domains of the subunits AF9 and ENL. Furthermore, H3K9ac functions in transcription to recruit the SEC to chromatin and facilitate subsequent pol II pause release, leading to increased transcription elongation as determined by pol II ChIP-seq and in vitro transcription assays.

**Results**

**H3K9ac recruits the SEC**

We previously demonstrated that the presence of H3K4me3 on nucleosomes promotes H3K9ac in a cell-free system (7). Additionally, the genome-wide co-occupancy of H3K4me3 and histone acetylation, especially H3K9ac, on active gene promoters has been widely reported in different cell types (16–19). To determine the functional role of H3K9ac associated with H3K4me3, we set out to identify possible readers of H3K9ac through a proteomics approach using liquid chromatography-mass spectrometry (LC-MS). We performed pulldown assays using biotinylated histone peptides and HeLa S-3 cell nuclear extract as a source of readers. Because we observed interplay between H3K4me3 and H3K9ac, we utilized peptides that either had H3K4me3 alone or both H3K4me3 and H3K9ac. Importantly, after analyzing proteins bound to these peptides that were identified by mass spectrometry, we were able to detect entire known H3K4me3 reader complexes recruited to H3K4me3 peptides, such as the general transcription initiation factor TFIIID and SAGA HAT complexes (supplemental Table 1).

We then specifically compared transcriptional protein complexes recruited to the dual modification H3K4me3-K9ac versus H3K4me3 alone. Strikingly, we observed all major subunits of the super elongation complex (AFF4, AFF1, AF9, ENL, ELL2, CDK9, and Cyclin T1/2) enriched on peptides containing H3K9ac as compared with control peptides (Fig. 1A), which we then validated by immunoblotting. However, analysis of the mass spectrometry data did not indicate other known pol II elongation factors to be enriched on H3K9ac, such as DOT1L, PAF1, Elongin, TFIIIF, and FACT complexes (Fig. 1B). Our validation experiments confirm that the SEC is recruited to H3K9ac alone in addition to the dual H3K4me3-H3K9ac mark, with reduced binding observed on the unmodified H3 or H3K4me3 peptides (Fig. 2, A and B). As observed by mass spectrometry, the TATA-box-binding protein-associated factor 1 (TAF1) subunit was detected on H3K4me3 peptides (likely through the reading action of TAF3) (22, 25) but absent on H3K9ac. The biochemical data suggest that the presence of an initiation mark (H3K4me3) may block binding of a specific elongation factor until the appearance of an additional acetylation mark to specify the correct “start time” for elongation. To determine whether the SEC also is recruited to nucleosomes containing H3K9ac, we assembled chromatin in vitro using recombinant histones and a biotinylated DNA fragment. Following nucleosomal DNA pulldown with streptavidin beads, we found that the SEC was enriched on nucleosomes containing H3K9ac compared with H3K4me3 (Fig. 2, C and D). When expanding the peptide pulldowns to include several additional acetylation marks on histones H3 and H4, we found that the SEC, including P-TEFB (CDK9 and Cyclin T1), is strongly recruited to H3K9ac and slightly enriched on H4K5ac (Fig. 2E). However, other acetylated peptides in the peptide pulldown do not recruit the SEC, but instead recruit other reader proteins, such as Brahma–related gene 1 (BRG1) to H3K14ac, which is consistent with previous reports (31, 32).

Because the SEC is recruited to H3K9ac and to a lesser extent H4K5ac, we next wanted to determine which reader proteins could be bridging the SEC to these acetylated lysines. A variety of potential reader proteins containing either Bromo or YEATS domains, previously characterized to recognize acetylated lysine residues (33, 34), were detected by LC-MS (Fig. 3A). We then employed an acetyl reader array in which GST-tagged Bromo and YEATS domains were immobilized on a plate and incubated with a Cy3-conjugated histone peptide. After being washed thoroughly, bound fluorescence was detected with a plate reader. Consistent with findings from Shi and co-workers (35), we found that the YEATS domain of AF9 showed the strongest binding to H3K9ac out of all the protein domains tested and did not bind to an unmodified H3 peptide (Fig. 3B). Unfortunately we could not determine direct readers from test-
ing the array with H4K5ac peptides compared with an H4 unmodified peptide, as the unmodified peptide had high back-
ground binding (Fig. 3 B).

We then wanted to confirm the direct binding of the AF9 YΕΑTS domain to H3K9ac. Using purified GST-tagged YΕΑTS domains in histone peptide pulldown assays, we found that the AF9 and ENL (both SEC subunits) YΕΑTS domains can bind to both H3K9ac and H4K5ac peptides, with lesser binding occurring on H4K5ac compared with H3K9ac (Fig. 4 A). This direct binding data show that a structurally defined domain can bind a particular acetylated histone residue, but it does not address the question of whether this interaction is important for recruit-
ment of the entire SEC complex. Additionally, because the protein bromodomain-containing 4 (BRD4) is known to interact with P-TEFb (36, 37), and contains bromodomains to directly bind to acetylated lysines, it is possible that the SEC may be recruited to H3K9ac and/or H4K5ac through “tethering” of bromodomain and extra-terminal (BET) family members. Although we did not observe enrichment of BRD4 or other BET family members on H3K9ac by mass spectrometry or direct binding of BET family members to H3K9ac in the acetyl binding array (Fig. 3), we still wanted to test whether these proteins could mediate SEC recruitment to H3K9ac or H4K5ac. We performed siRNA knockdowns targeting AF9 and ENL or BRD2, -3, and -4 in HeLa cells and made nuclear extracts for use in histone peptide pulldown assays. Compared with nuclear extract from cells transfected with the non-targeting control siRNA, knockdown of AF9 and ENL largely reduced the recruitment of the SEC on acetylated histone lysines compared with unmodified histone peptides (Fig. 4B). However, knockdown of BRD2, -3, and -4 had no substantial effect on SEC recruitment to either H3K9ac or H4K5ac (Fig. 4B).

Together, our biochemical data demonstrate that AF9 and ENL can specifically and directly bind H3K9ac for recruitment of the entire SEC complex, a result not reported previously. Based on the direct binding data in Fig. 4A, AF9 and ENL appear to have higher affinities for binding H3K9ac as compared with H4K5ac. This binding data suggest that these SEC subunits can specifically recognize these acetylation marks, and our knockdown data in Fig. 4B show that the SEC as a complex is being recruited to H3K9ac and H4K5ac through the action of AF9 and ENL. In sum, our data suggest that multiple acetyl marks on different histone tails may recruit the SEC via its YΕΑTS domain-containing subunits to chromatin, which may be important for productive transcription elongation.

**H3K9ac is required for maximal transcriptional pause release in vitro**

We next wanted to determine the direct impact of H3K9ac on transcription, and we chose to focus on H3K9ac, and not H4K5ac, as we observed stronger SEC binding to H3K9ac. To first obtain a clear mechanistic paradigm, we utilized a cell-free approach in which we could better control for the presence or absence of histone marks. We assembled chromatin with HeLa core histones and a DNA template containing four tandem estrogen-response elements (ERE) upstream of the adenovirus E4 gene promoter followed by a G-less cassette of 48 bp (4xERE-E4-G48). Using this template, we measured activated transcription in vitro using the estrogen receptor α (ERα) as a model activator and with HeLa S-3 nuclear extract providing the pol II transcriptional machinery, and we then detected tran-
scripts by RT-qPCR using primers downstream of the G-less cassette. By excluding GTP from the reaction, pol II will pause at G48, and this pause can be released by a simple addition of GTP. Transcription was induced in the presence of ERα and GTP compared with the absence of either (Fig. 5A). We then purified either wild-type H3 (WT) or mutant H3 K9R recom-
binant histones from bacteria and assembled chromatin using these histones plus recombinant H2A, H2B, H4, and the 4xERE-E4-G48 DNA. Importantly, WT and K9R histone H3 both yielded similar patterns of chromatin assembly when sub-
jected to micrococcal nuclease (MNase) digestion (Fig. 5B).
Unlike WT H3-containing chromatin that displays robust induced transcription after GTP addition, chromatin templates bearing H3 K9R displayed reduced transcription, implying a defect in pause release and resumption of transcription elongation (Fig. 5C).

To address whether this observed reduction in transcription was due to SEC action, we tested the effect of SEC immunodepletion from HeLa S-3 nuclear extract on activated transcription using nucleosomal templates assembled with either WT or K9R histone H3. After confirming immunodepletion of SEC using antibodies raised against different subunits (Fig. 5D), we tested these extracts for an effect on activated transcription. We specifically chose to use the extract after depletion with antibodies targeting CDK9 and ELL2, as this extract had the best depletion of CDK9, the enzymatic subunit of the SEC. On WT H3 chromatin, transcription with SEC-immunodepleted nuclear extract was significantly reduced compared with control IgG immunodepleted extract. However, we observed no significant difference on H3 K9R chromatin in transcription using SEC versus IgG immunodepleted nuclear extracts (Fig. 5E). These findings suggest that SEC immunodepletion reduces transcription in an H3K9-dependent manner.
GCN5/PCAF knockdown increases pol II promoter proximal pausing on a subset of genes

Based on our biochemical data that the SEC is recruited to H3K9ac and the published role of the SEC in transcriptional elongation, we hypothesized that modulating levels of H3K9ac in cells would impact pol II pausing. We chose to achieve this modulation of H3K9ac by targeting GCN5/PCAF for knockdown, as loss of these enzymes reduces global levels of H3K9ac.

### Table A

| Gene   | Pept Pept | Amt Amt | Fold Change |
|--------|-----------|---------|-------------|
| ASH1L  | 2         | 0.34    | 2           |
| ATAD2  | 1         | 0.01    |             |
| BAZ1A  | 7         | 0.39    | 7           |
| BAZ2B  | 1         | 0.02    | 1           |
| BPTF   | 28        | 7.1     | 30          |
| BRD4   | 1         | 0.05    | 1           |
| BRD7   | 4         | 0.63    | 4           |
| BRD8   | 3         | 0.13    | 3           |
| BRPF1  | 2         | 0.18    | 2           |
| BRPF3  | 2         | 0.05    | 2           |
| BRWD1  | 1         | 0.08    | 1           |
| BRWD3  | 1         |         |             |
| EP300  | 1         | 0.57    | 1           |
| KAT2A  | 17        | 7.9     | 17          |
| KAT2B  | 3         | 0.04    | 3           |
| PBRM1  | 24        | 4.8     | 23          |
| PHIP   | 2         | 0.21    | 2           |
| SMARCA2| 13        | 0.89    | 10          |
| SMARCA4| 22        | 8.2     | 20          |
| SP100  | 1         |         | 1           |
| SP140L | 2         | 0.68    | 2           |
| TAF1   | 22        | 5.6     | 21          |
| TAF1L  | 13        |         | 13          |
| TRIM24 | 2         | 0.05    | 2           |
| TRIM28 | 16        | 3       | 14          |
| TRIM33 | 3         | 0.05    | 3           |

### Table B

| Gene  | Pept Pept | Amt Amt | Fold Change |
|-------|-----------|---------|-------------|
| AF9   | 0         | 0       | 2           |
| ENL   | 6         | 1.5     | 6           |
| YEATS2| 5         | 0.08    | 5           |
| YEATS4| 5         | 13.4    | 6           |

Legend:
- 1:25-2: Fold change
- 2:5: Fold change
- 5:10: Fold change
- 10:20: Fold change
- 20:50: Fold change
- >50: Fold change

**Up:** Fold change

**Down:** Fold change
in HeLa cells (Fig. 6A), without affecting the expression of the reader protein, AF9. To functionally test our hypothesis, we performed ChIP-seq for total pol II occupancy after GCN5/PCAF knockdown in HeLa cells and calculated the pol II pausing index on a gene-by-gene basis. We then analyzed genes that had an altered pausing index by at least 1.5-fold in the siGCN5/PCAF samples compared with the NT-siRNA samples, and we found that 1,105 and 439 genes displayed increased and reduced pausing indexes, respectively (Fig. 6B; supplemental Table 3). Importantly, after analyzing these changes for significance across two independent replicates, we found that the vast majority of genes (240 genes, or 72%) with significantly altered pausing had a significantly increased pausing index after GCN5/PCAF knockdown, compared with a smaller number of genes that displayed reduced pausing (94 genes, or 28%) (Fig. 6B; three representative gene loci shown in Fig. 6C). These data provide important cell-based support to our hypothesis that histone acetylation, specifically H3K9ac, mediates pol II pausing on a subset of genes in HeLa cells.

We then utilized our ChIP-seq data to identify candidate genes to further test our hypothesis and perform additional experiments. We specifically focused on genes for which mRNA expression would be reduced upon knockdown of both GCN5 and PCAF, as well as the H3K9ac reader proteins AF9 and ENL. To first address the possibility that these knockdown experiments may impact the expression of each other, we analyzed the expression of AF9 and ENL after knockdown of GCN5/PCAF and vice versa. GCN5/PCAF knockdown had no effect on AF9 and ENL mRNA levels, and AF9/ENL knockdown did not affect expression of GCN5 and PCAF (Fig. 7A), consistent with our analysis of protein expression (Fig. 6A). Furthermore, in contrast to GCN5/PCAF knockdown, AF9/ENL knockdown did not affect global levels of H3K9ac (Fig. 6A). We then focused on genes that had both a high pausing index after GCN5/PCAF knockdown as well as AF9 occupancy (35) to perform additional mechanistic analysis. The genes PIM1, SGK223, SMAD3, KLHL22, ERRFI1, MKNK2, and RDX all displayed robust increases in pol II pausing index and had reduced expression after both AF9/ENL knockdown and GCN5/PCAF knockdown (Fig. 7B). Importantly, knockdown of all four transcripts (AF9, ENL, GCN5, and PCAF; termed “siALL”) did not further reduce expression of our candidate paused genes, suggesting AF9/ENL and GCN5/PCAF are acting in the same pathway. We therefore selected these genes for further mechanistic analysis by ChIP-qPCR.

**Reduction of H3K9ac decreases SEC occupancy and C-terminal domain of pol II (CTD) phosphorylation events on select target genes**

To next confirm our biochemical findings that the SEC is recruited to H3K9ac in living cells, we analyzed SEC occupancy on select cellular genes after modulating levels of H3K9ac. Importantly, Shi and co-workers (35) previously showed that GCN5/PCAF null mouse embryonic fibroblasts have reduced global AF9 occupancy on chromatin compared with wild-type mouse embryonic fibroblasts, suggesting that H3K9ac is necessary for AF9 localization. Compared with non-targeting siRNA control, GCN5/PCAF knockdown resulted in a loss of H3K9 acetylation at the proximal promoter regions of our candidate paused genes (PIM1, SGK223, SMAD3, KLHL22, ERRFI1, MKNK2, and RDX) as assayed by ChIP-qPCR, consistent with the global loss of H3K9ac (Fig. 8A). CDK9 (the enzymatic subunit of the SEC) occupancy was also reduced after GCN5/PCAF knockdown at the same region of these genes (Fig. 8B). The close correlation between these two events suggests that H3K9ac is important for SEC recruitment to chromatin in living cells, and this occupancy has functional effects on transcription as assayed by pol II pausing and gene expression analysis.

We next wanted to validate our ChIP-seq results on our candidate paused genes after reducing levels of H3K9ac or ablation of H3K9ac readers in living cells. We therefore examined total pol II occupancy at promoter proximal pause sites by ChIP-qPCR. Indeed, we found that pol II occupancy was enhanced directly downstream of the transcription start sites on our target genes after GCN5/PCAF knockdown in HeLa cells (Fig. 8C), despite reduced gene expression (Fig. 7B). Importantly, this pol II build-up was also observed after knockdown of AF9/ENL (Fig. 8D), consistent with previous reports in which knockdown of SEC subunits resulted in a build-up of pol II at select promoters in HeLa cells (38), suggestive of a defect in promoter clearance and/or pol II pause release.

In addition to examining total pol II occupancy, we wanted to determine how loss of H3K9ac would impact pol II CTD phosphorylation events at these genes. At the proximal promoter region, phosphorylation on serine 5 is typically associated with transcription initiation and promoter clearance through the action of cyclin-dependent kinase 7 (CDK7), and serine 2 phosphorylation is deposited by CDK9 and implicated in pol II pause release and the transition to productive elongation (39). Interestingly, phosphorylation on both serines 2 and 5 is reduced on our candidate paused genes after GCN5/PCAF knockdown (Fig. 8E). Although this result was initially surprising, phosphorylation on both serines has previously been implicated in regulating transcription elongation, and multiple kinases have been implicated in targeting the serine residues on the pol II CTD (40–42). CDK9 was first shown to phosphorylate serine 2 of the pol II CTD (27–30), but it also has been reported to phosphorylate serine 5 (43, 44).

Because we observed reduced phosphorylation on serines 2 and 5 of the pol II CTD, we wanted to determine whether GCN5/PCAF knockdown had any effect on pol II preinitiation...
complex (PIC) formation. Using antibodies to two components of the PIC, mediator of RNA polymerase II transcription subunit 12 (MED12) and TBP, we interrogated the occupancy of these proteins in the proximal promoter region of our candidate genes as a function of GCN5/PCAF knockdown. Importantly, knockdown of GCN5/PCAF had no effect on either MED12 or TBP occupancy (Fig. 8F), suggesting that loss of H3K9ac does not affect PIC formation at these gene targets. Together, these results provide important cellular support to our hypothesis, suggesting that histone acetylation, especially H3K9ac, is necessary for maximal pol II pause release at select gene promoters in HeLa cells.

Figure 4. AF9 and ENL recruit the SEC to H3K9ac peptides. A, YEATS domains of AF9 and ENL bind H3K9ac peptides. Purified GST-tagged recombinant YEATS domains were subjected to peptide pulldown followed by immunoblotting using a GST antibody. Representative immunoblots are shown, with quantification of immunoblotting replicates (right) representing raw band intensities detected by ImageJ software, n = 2. Vertical lines, range; horizontal lines, mean. B, knockdown of AF9 and ENL reduces SEC recruitment to H3K9ac and H4K5ac peptides. HeLa cells were transfected with either non-targeting siRNA (NT-siRNA), siRNA targeting AF9 and ENL (siAF9/ENL), or siRNA targeting BRD2, -3, and -4 (siBRD2/3/4). After 2 days, cells were harvested, and small-scale nuclear extract was prepared and then used in peptide pulldown assays, followed by immunoblotting. Representative immunoblots are shown, with quantification of immunoblotting replicates (bottom, right) representing raw band intensities detected by ImageJ software, n = 2. Vertical lines, range; horizontal lines, mean.
The histone code hypothesis suggests that the order and combination of histone marks may specify temporal downstream functions in a cell (6). Our studies add a further layer to histone-coded pol II transcription progression, as we have identified additional relationships between histone marks and the transcription cycle. Although multiple studies have demonstrated that there is an interplay between H3K4me3 and H3K9ac, and both are associated with gene expression and active transcription (7, 16, 17, 19), the functional significance of this interplay was less clear. We propose that the chromatin state allows transcriptional machinery to decode the status of a gene; the presence or absence, combination, and order of particular histone marks may indicate whether transcription has already occurred or whether a gene is primed for a particular stage of active transcription. Historically, histone acetylation was thought to be activating for transcription, as hyperacetylation was demonstrated to lead to a more loose chromatin structure (20). Although H3K9ac and other acetylation marks may influence chromatin structure, we hypothesized that these acetylation events could also recruit specific reader proteins for transcription progression. Indeed, we found that the SEC is recruited to H3K9ac (Figs. 3 and 4), suggesting that these acetylation marks may help direct progression of transcription into elongation. Because H3K4me3 recruits proteins essential for transcription initiation, including the TFIID complex through its TAF3 subunit (22, 25) and GCN5–PCAF HAT complexes that deposit acetylation on H3K9 (8, 9), we propose that H3K9ac helps to designate the transition from transcription initiation to elongation on chromatin.

We demonstrated that recruitment of the SEC to H3K9ac occurs through the direct reading capability of the AF9 and ENL YEATS domains (Figs. 3 and 4). This discovery establishes a new mechanism of SEC recruitment to chromatin, which we validated in cells by knockdown of GCN5/PCAF (Fig. 8). Previously, the SEC was shown to be targeted to genes through several mechanisms, including interactions with the Mediator complex at immediate early genes (45) and hypoxia-inducible genes (46). In the context of transcription of the HIV genome, the SEC is recruited to the viral promoter by the viral protein Tat (47). Therefore, the SEC may be anchored to chromatin through select acetylated histone tail residues and thus is distinct from its recruitment through previously known protein–protein interactions. Different histone acetylation marks may serve to reinforce recruitment of the SEC and function as an additional means of "fine-tuning" the targeting of SEC to chromatin. Multiple acetylated residues also could recruit multiple SEC complexes at a given promoter, result-

Figure 5. Loss of histone H3K9 acetylation reduces transcription after pol II pause release in vitro. A, G-less cassette template 4xERE-E4-G48 is activated by ERα in a GTP-dependent manner. 4xERE-E4-G48 was assembled into chromatin with HeLa core histones in the presence or absence of ERα and GTP. mRNA was measured by RT-qPCR using primers downstream of the G-less cassette. ***, p value < 0.001 by one-way ANOVA followed by adjusting for multiple comparisons. B, chromatin assembly with wild-type (WT) or K9R H3.3 histones results in similar chromatin assembly. Chromatin was assembled with WT or K9R histone H3 and then subject to MNase digestion followed by agarose gel electrophoresis and staining with ethidium bromide. Positions of nucleosomes are indicated with arrows. C, loss of H3K9 reduces pol II pause release from a GTP-deprivation stalled template in vitro. mRNA was measured by RT-qPCR using primers downstream of the G-less cassette. *, p value < 0.05 by Student’s t test. D, validation of SEC immunodepletion (Δ) from HeLa S-3 nuclear extract using different antibodies targeting the SEC. Rabbit IgG, AFF1 and AFF4, or CDK9 and ELL2 antibodies were used for immunodepletion from HeLa S-3 nuclear extract, which was confirmed by immunoblotting. E. histone H3.3 K9R mutant phenocopies the effect of SEC immunodepletion on GTP-pause released pol II transcription. Transcription was carried out as described using the ΔCDK9/ELL2 (ΔSEC) extract. *, p value < 0.05. Statistical significance was determined using a one-way ANOVA followed by adjusting for multiple comparisons.
ing in greater rates of pause release and elongation for multiple rounds of transcription.

Because the SEC has an established function in promoting transcription elongation (26, 48) and we found SEC binding to H3K9ac, we hypothesized that select histone acetylations may impact transcription elongation through SEC recruitment. Transcription elongation is a critical gene regulatory step that has gained greater appreciation from data published from numerous laboratories employing both ChIP-seq and global run-on sequencing (GRO-seq). In these studies, widespread pausing was observed on transcribed mammalian genes (49–51). It is now estimated that the majority of mammalian pol II genes can be regulated at the transition from transcription initiation to elongation (2–4). Here, we propose that a single acetyl residue on one histone tail can have significant effects on transcriptional pause release through recruiting reader proteins, as demonstrated by our *in vitro* transcription results in which mutating H3K9 to H3 K9R resulted in significantly decreased transcription after GTP-mediated release of paused pol II complexes (Fig. 5). Additionally, our cellular modulation
of H3K9ac levels through the knockdown of GCN5 and PCAF provides further support to our hypothesis, as loss of H3K9ac significantly increases the pol II pausing index on a subset of genes in HeLa cells, without affecting all transcriptional machinery (Figs. 6–8).

Although the release of pol II from a paused state is a regulatory step on many genes and H3K9ac is found at the majority of actively transcribed promoters, our data suggest that the recognition of H3K9ac via SEC binding applies to a subset of genes in asynchronous HeLa cells. Loss of H3K9ac resulted in significantly increased pol II pausing indexes on 240 genes and decreased pausing indexes on 94 genes, as assayed by pol II ChIP-seq. We propose several possible reasons for why only a subset of genes displays increased pol II pausing, and why a smaller subset of genes displays decreased pol II pausing. First, H3K9ac could have multiple functional roles in the cell, and we are simply describing only one of those functions relevant to transcription. Second, our study has focused on basal pol II transcription in HeLa cells, and it is possible that this mechanism may be more utilized in response to environmental stimuli or regulatory cues that have yet to be elucidated. Third, we analyzed pol II pausing after knocking down the writers of H3K9ac, which may have additional consequences in the cell, as GCN5 and PCAF play important roles in a variety of cellular processes (52, 53). Finally, pol II pausing is regulated by a number of factors and is not only dependent on the SEC (3).

In summary, our data indicate that histone H3 acetylation at lysine 9 may mediate recruitment of the SEC to chromatin to transition from transcription initiation to elongation through the release of paused pol II (Fig. 9). Additional histone acetylations, such as H4K5ac, may further recruit and/or stabilize the SEC to target gene promoters bearing H3K9ac. Although H3K4me3 recruits protein complexes that are essential for transcription initiation, subsequent acetylation events may directly target the SEC to chromatin on particular genes. This recruitment of the SEC to acetylated histone lysines occurs through direct binding of its YEATS domain-containing sub-

Figure 7. Loss of H3K9ac writers or readers reduces expression of select pol II paused genes. HeLa cells were transfected in triplicate with siRNA targeting GCN5/PCAF or AF9/ENL and compared with a non-targeting (NT-siRNA) control. Gene expression was analyzed using the ΔΔCT method with 18S rRNA as an endogenous control. Statistical significance was determined using a one-way ANOVA followed by adjusting for multiple comparisons, and p values less than 0.05 were considered significant. A, knockdown of GCN5/PCAF does not affect AF9 or ENL mRNA levels, and AF9/ENL knockdown does not affect GCN5 or PCAF mRNA levels. ***, p value < 0.001. B, select paused genes display reduced gene expression after GCN5/PCAF or AF9/ENL knockdown. ***, p value < 0.001; **, p value < 0.01; *, p value < 0.05. Legend is the same as in A.
Figure 8. H3K9ac mediates SEC and pol II chromatin occupancy on the proximal promoter region of select genes. HeLa cells were subject to ChIP-qPCR analysis following transfection of siRNAs, and ChIP assays were repeated at least three independent times. Representative results are shown. Knockdown of GCN5/PCAF results in reduced H3K9ac occupancy (A) and decreased CDK9 occupancy (B). Knockdown of GCN5/PCAF (C) or AF9/ENL (D) results in a build-up of total pol II occupancy. E, GCN5/PCAF knockdown decreases phosphorylation at both serine 2 (pSer2) and serine 5 (pSer5) on the pol II CTD. ChIP was performed using pol II CTD phosphorylation-specific antibodies. F, Mediator and TBP occupancies are not affected by GCN5/PCAF knockdown. ChIP was performed using antibodies targeting MED12, TBP, and IgG as a negative control. IP, immunoprecipitation; Neg, negative; Ctrl, control.
units, AF9 and ENL. In this manner, H3K9ac can direct the next step of transcription in which paused pol II is released and productive elongation ensues.

**Experimental procedures**

**Reagents**

Antibodies used in this study are listed below with source, catalogue number, dilution used in immunoblotting (if applicable), and amount used in ChIP (if applicable). Antibodies from Active Motif were as follows: total histone H3 (39163, 1:5,000, 5 μg for ChIP); H3K9ac (39917, 1:2,000, 5 μg for ChIP); and Ser(P)-5 pol II CTD (61085, 5 μg for ChIP). Antibodies from Bethyl Laboratories were as follows: AF9 (A300–595A, 1:2,000); AFF1 (A302–345A, 1:2,000); AFF4 (A302–539A, 1:4,000); BRD2 (A302–582A, 1:1,000); BRD3 (A302–368A, 1:2,000); BRD4 (A301–985A50, 1:500); BRG1 (A300–813A, 1:1,000); Cyclin T1 (A303–499A, 1:2,000); ELL2 (A302–505A, 1:2,000); ENL (302–268A, 1:2,000); and MED12 (A300–774A, 5 μg for ChIP). Antibodies from Santa Cruz Biotechnology were as follows: CDK9 (sc-8338, 1:200, 5 μg for ChIP); GCN5 (sc-20698, 1:200); p300 (sc-584, 1:200); pol II (sc-899, 2–3 μg for ChIP); TAF1 (sc-735, 1:200); and TBP (sc-273, 5 μg for ChIP). The β-actin antibody was from Sigma (A1978, 1:5,000); the IgG antibody was from Millipore (12-370, 5 μg for ChIP), and the GST antibody was from Millipore (12-370, 5 μg for ChIP), and the GST antibody was from Millipore (12-370, 5 μg for ChIP).

Chromatin assembly

Chromatin assembly was performed as described previously (7). Briefly, the recombinant *Drosophila ACF1/ISWI/NAP-1* assembly system (59) was used for assembly of recombinant histones (New England Biolabs), modified histones (Active Motif), or HeLa core chromatin (Active Motif) onto the 4xERE-E4 (7) or the 4xERE-E4-G48 biotinylated fragment. The quality of nucleosome assembly was checked by MNase partial digestion using the Chromatin Assembly Kit from Active Motif.
followed by agarose gel electrophoresis and ethidium bromide staining.

### Pulldown assays

Either 120 or 60 μl of Dynabeads® M280 streptavidin beads (Invitrogen) were used to immobilize biotinylated histone peptides (Epicypher) or chromatinized DNA pulldowns, respectively. 5 μg of peptides in 150 μl of D-PBS or 4 μg of chromatinized 4×ERE-E4 DNA were bound to the Dynabeads by rotation for ~1 h at 4 °C and then washed once with D-PBS. Beads were resuspended in 1 mg of clarified HeLa S-3 nuclear extract (prepared as described (7)) or with small-scale nuclear extracts made from HeLa cells transfected with siRNA (see below) and supplemented with 1 mM EDTA/EGTA. Reactions were incubated with rotation at 4 °C for 1.5 h. Beads were washed as described and were resuspended in 20–30 μl of 2× SDS sample buffer (Pierce). For pulldown assays using recombinant proteins, 2.5 μg of histone peptides were bound to 60 μl of streptavidin beads, and 3 μg of protein was added in 150 μl of binding buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1 mM PMSF). Beads were washed a total of five times: twice with binding buffer, followed by twice with NETN (as described previously (7)), and finally once with D-PBS. After boiling, protein samples were loaded on to 4–15% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad) for immunoblotting or on NuPAGE gels (8% BisTris in MOPS buffer, Invitrogen) for preparative separation-gel slice excision for mass spectrometry.

### Small-scale nuclear extracts

These extracts were made from siRNA-transfected HeLa cells as follows: D-PBS-washed cell pellets were resuspended in hypotonic buffer (7) containing 0.5 mM DTT and protease inhibitors (Roche Applied Science), allowed to swell on ice for 10 min, and then snap-frozen in liquid nitrogen. After the cell suspensions were thawed on ice, nuclei were pelleted at 3,500 g for 10 min at 4 °C and then resuspended in ½ packed nuclei volume (pnv) of low salt buffer containing 0.5 mM DTT and protease inhibitors. Nuclei were extracted at 4 °C for 15 min after addition of ½ pnv of high salt buffer containing 0.5 mM DTT and protease inhibitors. The low salt and high salt buffers were made as described previously (60). Final nuclear extract was diluted with an equal volume containing 0.5 mM DTT and protease inhibitors to reduce the salt concentration to ~200 mM.

### Mass spectrometry sample preparation and nano LC-MS/MS analysis

Washed beads were boiled in 30 μl of 1× NuPAGE® LDS sample buffer (Invitrogen) and subjected to SDS-PAGE (NuPAGE 10% BisTris gel, Invitrogen). The eluted proteins were visualized with Coomassie Brilliant Blue stain and excised into six gel pieces according to molecular size. The individual gel pieces were destained and subjected to in-gel digestion using trypsin. The tryptic peptides were resuspended in 10 ml of loading solution (5% methanol containing 0.1% formic acid) and subjected to nanoflow LC-MS/MS analysis with a nano-LC 1000 system (Thermo Fisher Scientific) coupled to LTQ Orbitrap Elite™ (Thermo Fisher Scientific) mass spectrometer. The peptides were loaded onto a Reprosil-Pur Basic C18 (1.9 μm, Dr. Maisch GmbH, Germany) pre-column of 2 cm × 100 μm size. The pre-column was switched in-line with an in-house 50-mm × 150-μm analytical column packed with Reprosil-Pur Basic C18 equilibrated in 0.1% formic acid/water. The peptides were eluted using a 75-min discontinuous gradient of 4–26% acetonitrile, 0.1% formic acid at a flow rate of 600 nl/min. The eluted peptides were directly electro-sprayed into LTQ Orbitrap Elite mass spectrometer operated in the data-dependent acquisition mode acquiring fragmentation spectra of the top 50 strongest ions.

### Proteomics database search and data validation

Obtained MS/MS spectra were searched against a target-decoy human refseq database in Proteome Discoverer 1.4 interface (Thermo Fisher Scientific) using lenient 1 or 5% strict or relaxed peptide-specific matches and false discovery rate (FDR), respectively. The data were then grouped into gene products and assigned homology and identification quality groups using an in-house developed algorithm. All protein gene products were required to have at least one identification where a spectral match passing <1% FDR and >20 ion score or >5% FDR and >30 ion score thresholds was present to be chosen for follow-up in this study. Each gene product amounts were estimated using a label-free intensity-based absolute quantification (iBAQ) approach (as the sum of peptide areas normalized to the theoretical tryptic peptide potential (61)) and then reported as a fraction of total protein iBAQ amount per experiment (in 10⁻⁵ units for visual comprehension).

### Immunoblotting

For analysis of proteins from HeLa cells, cells were scraped in PBS and resuspended in NETN with 10% glycerol and protease inhibitors. Protein samples were transferred from SDS-polyacrylamide gels to PVDF (Bio-Rad) as described previously (7). Ponceau S (Sigma) staining was performed to assess transfer efficiency before blots were cut into appropriate strips of different size ranges. Blots were blocked, incubated with antibodies (listed in under “Reagents”), and developed using enhanced ECL (Thermo Fisher Scientific) and X-ray film as described previously (7).

### Protein array experiments

A set of human Bromo and YEATS protein domains was cloned into a pGEX vector. Cloning was performed by BioMatik (Cambridge, Canada) using gene synthesis to codon optimize the open reading frames for bacterial expression. All domains were expressed as GST fusions in Escherichia coli and purified on glutathione-Sepharose beads. The recombinant domains were arrayed onto nitrocellulose-coated glass slides (Onyx®vid slides, Grace Bio-Labs, Bend, OR), using an Aushon 2470 pin microarrayer as described previously (62). A list of the recombinant domains on this array is provided as supplemental Table 2. The fluorescent labeling of the biotinylated peptide probe and slide binding have also been previously described (62). Fluorescent signal was detected using a GeneTACTM LSIV scanner (Genomic Solutions).
**siRNA transfections**

HeLa cells (from BCM Tissue Culture Core via ATCC) were plated for immunoblotting, mRNA analysis, or ChIP in complete media (DMEM, 10% FCS, 1% penicillin/streptomycin). Cells were transfected using RNAiMAX (Invitrogen) with siGENOME SMARTpool siRNA from Dharmacon targeting GCN5 (M-009722-01-0005), and PCAF (M-005055-00-0005), or a non-targeting siRNA (pool 2, D-001206-14-05) at a final concentration of 100 nM. Cells were harvested after 72 h of total siRNA treatment. The same protocol was followed for siRNAs from Dharmacon targeting AF9 (M-019813-01-0005), ENL (M-019813-01-0005), BRD2 (M-019813-01-0005), BRD3 (M-004936-01-0005), and BRD4 (M-004936-01-0005), except cells were transfected with 60 nM total siRNA and harvested after 48–72 h of transfection.

**RNA isolation and analysis**

RNA was extracted from cells plated in triplicate using TRIzol (Invitrogen) and purified using phenol/chloroform extraction. To measure the relative mRNA levels, real-time quantitative reverse transcription-PCR (RT-qPCR) was performed in a Step One Plus fast real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green (Invitrogen) reagents. Average threshold cycle (Ct) values of an endogenous housekeeping gene (18S rRNA) were subtracted from corresponding average Ct values of a target mRNA to obtain ΔCt values. Relative mRNA levels were expressed as $2^{-\Delta\Delta Ct}$ compared with the non-targeting siRNA control (63). Statistical significance was determined using the Student’s t test or ANOVA followed by adjusting for multiple comparisons using the Dunnett method, and $p$ values < 0.05 were considered significant. The primers for gene expression analysis are listed in supplemental Table 4.

**Histone extraction**

Cells were pelleted and washed in D-PBS and then resuspended in NETN with 10% glycerol and protease inhibitor mixture (Roche Applied Science). After a 30-min incubation at 4 °C rotating, the lysate was pelleted. The supernatant was saved as cell lysate, and the pellet was subject to TCA/acetone extraction (64). Briefly, the pellet was resuspended in nuclease-free water and treated with an equal volume of 25% TCA (Sigma). Following a 15-min incubation on ice, the samples were pelleted and incubated in ice-cold acetone (Sigma) for 20 min. The samples were pelleted and allowed to air-dry before resuspending in 2× SDS sample buffer (Pierce) with 10% Tris-HCl, pH 8.0 (Invitrogen). Samples were then boiled for 5 min, followed by centrifugation at full speed for 10 min. Samples of the supernatant were run on an SDS-polyacrylamide gel and stained using InstantBlue (Expedeon) to quantify histones for immunoblotting.

**Chromatin immunoprecipitation (ChIP)-seq**

ChIP-seq was performed by Active Motif (Carlsbad, CA). HeLa cells were subject to siRNA transfections as described above, then cross-linked, and harvested according to the Active Motif protocol. ChIP was performed targeting total pol II using an Active Motif antibody (catalogue number 39097) in biological duplicate reactions. Between 21 and 27 million reads per sample were mapped to the human genome hg19 using bowtie (version 1.1.0) (65). Only uniquely mapped reads with up to one mismatch were retained for further analysis. After subtracting the input, pol II read densities at the TSS proximal region (~30 to +300 bp around the TSS) and gene body (+300 bp downstream of the TSS to the transcription end site) were normalized as RPKM according to previous studies (66, 67). To obtain high quality results, only genes with RPKM >1 at both regions were used for calculating the pausing index. The pol II pausing index was defined as the RPKM at TSS proximal region divided by RPKM at the gene body of the same gene, and pausing indexes were then compared between the NT-siRNA control samples and siGCN5/PCAF samples. Genes with changed pausing indexes of at least 1.5-fold were determined using Student’s t test, and $p$ values of less than 0.05 were considered significant. The GEO accession number for this ChIP-seq data is GSE99998.

**ChIP-qPCR**

ChIP-qPCR assays were performed using the EZ ChIP kit (Millipore) per the manufacturer’s instructions. Briefly, cells in 15-cm dishes were cross-linked in 1% formaldehyde (Sigma) in D-PBS for 10 min and quenched with 125 mM glycine for 5 min. Chromatin was sheared by sonication using a microtip probe sonicator (Branson). Each sample was subjected to overnight IP with magnetic protein G Dynabeads (Invitrogen), followed by washes, elution of DNA–protein complexes, cross-link reversal, and spin-column cleanup. Purified DNA was used in qPCR reactions using SYBR green reagents on a Step One Plus machine (Applied Biosystems). All ChIP experiments were repeated at least three times and representative results are shown. See supplemental Table 5 for ChIP-qPCR primers.

**Recombinant YEATS domain purification**

GST-tagged YEATS domains were cloned into pGEX plasmids at Biomatik (Cambridge, Canada). Plasmids were transformed into Rosetta (DE3) competent cells (Novagen), and cultures were grown in Luria broth (LB) with ampicillin for selection and treated with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at room temperature to induce recombinant protein production. The cultures were pelletted, and cell pellets were lysed by sonication (for 1 min, six times) on ice in Buffer A (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 M NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, and 1 mM PMSF). After centrifugation, the supernatant was added to equilibrated GST beads (GE Healthcare). After washing with Buffer A and 0.1× Buffer A, proteins were eluted with 10 mM glutathione (Calbiochem) in 50 mM Tris-HCl, pH 8.0. Following purification, the proteins were analyzed for purity and yield by SDS-PAGE followed by staining with InstantBlue (Expedeon) to visualize the protein and quantified with Bradford reagent (Bio-Rad).

**Recombinant histone purification and mutagenesis**

The human histone H3.3 gene H3F3A was cloned into the pET-16b expression vector (Novagen). Site-directed mutagenesis was performed using the QuikChange Lightning kit (Agilent).
H3K9ac mediates Pol II pause release

We received the G-less cassette pG5-E4 array (68) as a generous gift from Dr. Jeffrey Parvin. We amplified the adenovirus E4 promoter and G-less region using PCR with High Fidelity Taq DNA polymerase (Invitrogen), digested the fragment with XbaI and StyI restriction enzymes (their sites were introduced in the PCR primers), and ligated the cut fragment into similarly digested pERE (7) using T4 DNA ligase (New England Biolabs). Successful generation of the desired plasmid was confirmed by sequencing. An 848-bp 4xERE-E4-G48 fragment was generated by PCR using Taq DNA polymerase (Invitrogen), digested the fragment with 4xERE and E4BioR primers as described previously (7), and the pERE-G48 plasmid. After PCR, any free primers were removed by passage through PCR Kleen Spin Columns (Bio-Rad).

In vitro transcription

In vitro transcription was performed as described previously (7, 69) with the following modifications for the 4xERE-E4-G48 construct. Transcription was initiated by the addition of ribonucleoside triphosphates (0.625 mM final) with or without GTP in a 50-µl final reaction mixture. Templates were transcribed at 30 °C for 50 min, and the synthesized RNA was extracted using TRI-Reagent (Molecular Research Center) and purified using Direct-zol RNA mini prep kits (Zymo Research). Turbo DNase (Ambion) was used to remove any residual DNA in the RNA preparation before further quantitative analysis of E4 mRNA by RT-qPCR. The primers used were 5′-GGATATGATATCGGCACAAC-3′ and 5′-GAGTCACTGAGCGAGGAGGAGC-3′. SensiFast One-Step SYBR Master Mix (BioLine) was used for qPCRs. All reactions were executed in triplicate, and each experiment was performed at least twice.

Immunodepletions

Immunodepletions of HeLa S-3 nuclear extracts were performed as described previously (7). 20 μg total of antibodies (rabbit IgG, a combination of ELL2 and CDK9, or a combination of AFF1 and AFF4) were bound overnight to BSA-blocked protein G-Sepharose beads (GE Healthcare). 3 mg of nuclear extracts were subject to two rounds of immunodepletion, which was confirmed by immunoblotting.

Statistical analysis

All statistical analysis was performed using the Student’s t test (unpaired, parametric, and two-sided) when comparing two means and ANOVA (ordinary, one-way) with correcting for multiple comparisons using the Dunnett method when comparing multiple means. Error bars on bar graphs represent standard deviations around the mean.

Author contributions—L. A. G. conducted the pulldown assays, siRNA knockdown experiments, and in vitro transcription. J. S. and W. L. performed analysis on sequencing data. L. A. G., A. D. R., and B. Z. conducted ChIP experiments. Q. F. provided support on protein purification and assay development. M. T. B. and C. A. S. conducted the protein array experiments. S. Y. J. and J. Q. conducted all mass spectrometry experiments. The manuscript was drafted by L. A. G. and edited by all authors. S. Y. T. and M. J. T. provided technical oversight and helpful discussion, and C. E. F. and B. W. O. provided conceptual framework and technical oversight on all experiments.

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Note added in proof—In the version of this article that was published as a Paper in Press on July 17, 2017, Fig. 6A contained an unintentional cropping error. This error has now been corrected and does not affect the results or conclusions of this work.

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