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# Actin associates with actively elongating genes and binds directly to the Cdk9 subunit of P-TEFb

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**Nuclear actin has been demonstrated to be essential for optimal transcription, but the molecular mechanisms and direct binding partner for actin in the RNA polymerase complex have remained unknown. By using purified proteins in a variety of biochemical assays, we demonstrate a direct and specific interaction between monomeric actin and Cdk9, the kinase subunit of the positive transcription elongation factor b required for RNA polymerase II pause-release. This interaction efficiently prevents actin polymerization, is not dependent on kinase activity of Cdk9, and is not involved with releasing positive transcription elongation factor b from its inhibitor 7SK snRNP complex. Supporting the specific role for actin in the elongation phase of transcription, chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) reveals that actin interacts with genes only upon their active transcription elongation. This study therefore provides novel insights into the mechanisms by which actin facilitates the transcription process.**

Actin is an abundant protein having essential roles both in the cytoplasm and in the cell nucleus. It is a key component of the cytoskeleton, but inside the nucleus, its functions are mostly related to gene expression control and maintenance of genomic integrity (1). The basic unit of actin is the globular monomer (G-actin) that can polymerize into filaments (F-actin) and the proper balance of these forms, maintained by actin-binding proteins (ABPs), is critical for actin functions. Shuttling of actin between the cytoplasmic and nuclear compartments is an active process and the two pools of actin are dynamically connected. Actin is imported into the nucleus by Importin-9 (Ipo9) together with small ABP cofilin (2) and exported by Exportin-6 (Exp6) as a complex with ABP profilin (3). Nuclear actin is an important regulator of gene expression. G-actin is a stoichiometric subunit of several chromatin remodeling and modifying complexes, which control the structure and accessibility of chromatin (4). Actin also directly regulates the activity of specific transcription factors, such as myocardin-related transcription factors (MRTFs) that are co-activators of the serum response factor (SRF). G-actin binding to MRTF prevents SRF activation (5), and there are various mechanisms to regulate MRTF/SRF pathway *via* altering actin nucleo-cytoplasmic transport and polymerization status (6).

One of the essential functions of nuclear actin is its role in transcription regulation. For optimal transcription, the levels of actin inside the nucleus have to be controlled, as manipulating actin levels impairs transcription both *in vitro* and in cells (2, 7–9). Decreased nuclear actin levels in response to laminin-111 and consequent PI3 kinase pathway attenuation leading to increased Exp6 activity have also been shown to destabilize Pol II and Pol III interactions with nuclear substructures, causing lower RNA synthesis rates and quiescence in mouse epithelial cells (10, 11). In *Drosophila* ovaries, actin and Pol II co-occupy promoter regions of most transcribed genes, as well as the gene bodies of highly transcribed genes (9). It is becoming clear that actin has functions throughout the transcription process. Actin has been linked to pre-initiation complex formation (12) and F-actin seems to cluster Pol II on inducible genes (13), promoting the formation of the transcription machinery. Actin also functions in the elongation phase and in Pol I–mediated transcription also nuclear myosin I is suggested to be involved, possibly combining the motor function of myosin with actin polymerization (8, 14, 15). Furthermore, actin has been linked to Pol II pause-release (16), an important control point of transcription by Pol II, in which the polymerase pauses shortly after elongation has started and needs positive transcription elongation factor b (P-TEFb) to proceed into productive elongation (17). In the transcription elongation phase, actin also associates with heterogeneous nuclear ribonucleoprotein U (18), and the function of actin here might be to recruit transcription coactivators, such as PCAF histone acetyltransferase (19). Finally, actin takes part in pre-mRNA processing, as previous results from our lab show that manipulating actin disturbs alternative splicing, either through a direct role in splicing or by affecting Pol II elongation or pausing rate so that also weaker splicing sites could be used (20). Altogether, there seems to be distinct roles for both monomeric and filamentous actin in transcription, and some interactions, in which the form of actin is not yet known.

Despite vast evidence of actin functions in transcription, the direct binding partner of actin within the RNA polymerases is not known. Actin has been suggested to co-purify with all

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three RNA polymerases (Pol): Pol I (21), Pol II (22, 23), and Pol III (7) and was already in the 1980's indicated to be involved in the transcription of protein coding genes (24). However, the form of actin associating with the polymerases is not known and the interaction remains to be characterized at a molecular level. Our mass spectrometry screen for nuclear actin interactome contained several transcription-related proteins as hits with BioID technique, detecting more transient interactions, but not in AP-mass spectrometry (MS) that is best suited for stable binding events (20). Also, most of the same proteins were captured with an actin mutant incapable of polymerization, suggesting that F-actin formation would not be needed for these interactions to take place. Among the BioID hits were both subunits of the P-TEFb complex (20). Here we demonstrate that actin, in its monomeric form, binds directly and specifically to the P-TEFb subunit Cdk9. We further demonstrate that actin associates with genes only upon their active elongation and that the kinase activity of Cdk9 is neither required for the interaction with actin nor actin recruitment to the transcribing genes. Functionally, actin is not involved in releasing P-TEFb from its inhibitory complex and modestly influences Cdk9 kinase activity.

## Results

### Actin is recruited to the genes only upon active transcription elongation and does not directly interact with the RNA polymerase II

Previous studies have implicated actin in several steps of transcription (12, 16, 18–20). By using chromatin immunoprecipitation followed by deep-sequencing (ChIP-seq), we have demonstrated that actin interacts with all transcribed genes near transcription start sites (TSS) in *Drosophila melanogaster* ovaries. In addition, we showed that actin accumulates on gene bodies of highly transcribed genes required for egg shell formation during *Drosophila* oogenesis (9). Heat shock protein (*hsp*) genes encode chaperone proteins that are required to maintain proteostasis not only during proteotoxic stress, such as heat shock (HS), but also during normal conditions. Indeed, many *hsp* genes are expressed also during *Drosophila* oogenesis (25). To study, if actin is binding to *hsp* genes *in vivo*, we used ChIP-seq on fly ovaries at room temperature (25 °C). Similarly, as we have reported earlier for the *chorion* genes (9), actin was found on the gene body of *Hsp68* (Fig. 1*A*). RNA polymerase II phosphorylated on serine 5 (Pol II S5P), peaked near the TSS, but was also abundant on the gene body (Fig. 1*A*). Analysis of the 15 *hsp* genes revealed similarly that Pol II is more concentrated near the TSS than actin, which displayed a rather broad distribution centered on the coding region of the gene (Fig. 1*B*). Thus, actin and Pol II do not display identical binding patterns on the *hsp* genes. Moreover, similarly as we reported earlier for *chorion* genes, RanBP9 mutant ovaries, which display reduced nuclear actin (9), show decreased occupancy of both actin and Pol II on the *hsp* genes (Fig. S1*A*). This indicates that balanced nuclear transport of actin is critical also for *hsp* gene transcription.

Since *hsp* genes are already abundantly expressed in fly ovaries without HS, we turned to the cultured fly cell line S2R+ as a model to study robust HS-induced transcriptional activation and performed ChIP-seq of actin and Pol II in cells cultured at room temperature (non-heat shock) or heat shocked for 20 min at 37 °C (Fig. 1, *C–E*). In non-heat shock conditions, Pol II is found mainly paused near the TSS of *Hsp68* gene (Fig. 1*C*). However, actin did not display any significant enrichment on the *Hsp68* gene (Fig. 1*C*) under these conditions, when the gene is not being actively transcribed. When the cells were heat shocked, Pol II was strongly recruited to the *Hsp68* gene and similarly as in fly ovaries, the distribution peaked near the TSS. Nevertheless, enrichment at the gene body and after the cleavage and polyadenylation site was also very evident (Fig. 1*C*). Upon HS, and similarly as observed in fly ovaries, actin was enriched on the coding region of *Hsp68* but did not show particular enrichment near TSS (Fig. 1*C*). Analysis of the 15 *hsp* genes revealed the same pattern (Fig. 1, *D* and *E*). Actin is not significantly associated with the *hsp* genes prior to their transcriptional activation by HS but only accumulates to the genes upon their active transcription elongation (Fig. 1*E*). The binding patterns of Pol II and actin are not similar (Fig. 1, *D* and *E*), indicating that actin does not interact with all Pol II complexes.

In several earlier studies, actin has been shown to copurify with the RNA polymerases (7, 21–23), but the details of this interaction have remained uncharacterized. We purified whole, transcriptionally active human Pol II from HeLa nuclear pellets to gain further insight into the possible interaction. However, in our Western blot of samples taken during the purification process (Fig. S1*B*), actin was present in the first steps of the purification but was lost during anion exchange (samples 3–4), indicating that the interaction is not stable and that actin is not an integral component of the purified polymerase, since it is not found in the final protein preparation (Fig. S1*B*, sample 8). We then studied, if the purified proteins would form a complex that would persist in size-exclusion chromatography (SEC) using buffer conditions optimal for actin and NaCl concentration of 100 mM to improve the performance of the gel filtration column. In this experiment, actin was complexed with Latrunculin B (LatB), a small molecule inhibiting actin polymerization that would otherwise take place when salt is present. The two proteins eluted from the SEC column in different fractions (Fig. S1*C*), which means that no complex was formed. This experiment suggests that actin, in its monomeric form, cannot directly interact with Pol II. This finding is further supported by our MS screen for nuclear actin-binding partners, which did not identify any Pol II subunits as high-confidence interactors in either affinity-purification-MS or BioID experiments (20).

### Actin monomers bind directly to the Cdk9 subunit of P-TEFb

Our ChIP-seq data demonstrated that actin bound only to actively elongating genes (Fig. 1, *C* and *E*), suggesting that a protein specifically required for transcription elongation may recruit actin to the transcribing polymerase. Previous studies

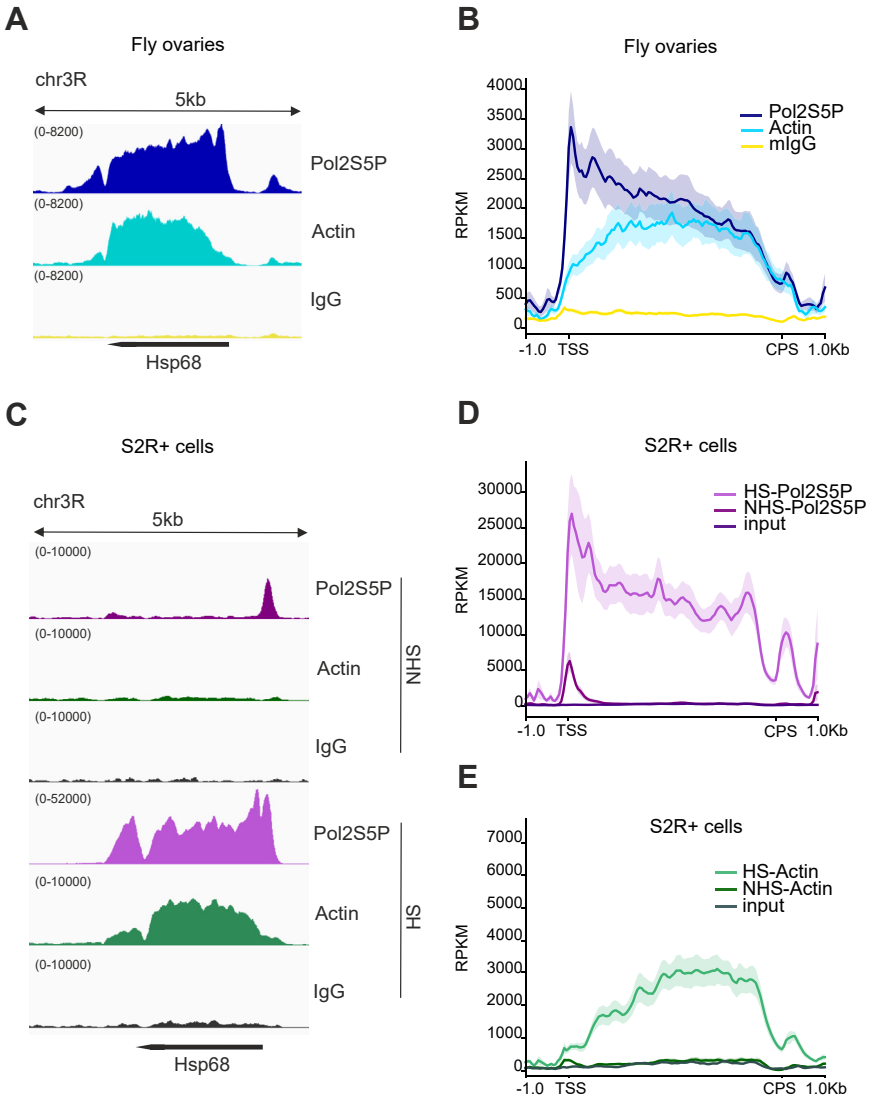


**Figure 1. Actin associates with *hsp* genes only upon their active transcription elongation.** *A*, actin binds *hsp* genes in *Drosophila* ovaries. Normalized (RPKM) coverage of RNA polymerase phosphorylated on serine 5 (Pol2S5P), actin, and normal IgG from chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) on *Hsp68* in *Drosophila* ovaries (W1118). *B*, Pol II S5P, actin, and mouse IgG coverage on 15 heat shock responding genes in *Drosophila* ovaries. Normalized fragment counts are scaled to 5 kb gene size. Transcription start site (TSS) and cleavage and polyadenylation site (CPS). *C*, actin binds to *hsp* genes only upon their transcriptional activation during heat shock (HS). Normalized (RPKM) coverage of Pol II S5P, actin, and normal IgG on Hsp68 from ChIP-seq in S2R+ cells in non heat shock (NHS) and HS (20 min) conditions. *D*, Pol II S5P coverage on 15 HS responding genes in S2R+ cells in NHS and HS conditions. Input is used as a negative control and data shown as in (*B*). *E*, actin coverage on 15 HS responding genes in S2R+ cells in NHS and HS conditions, shown as in (*D*).

had demonstrated that actin can be co-immunoprecipitated with P-TEFb (16), which plays a critical role in releasing the paused polymerase to active transcription elongation. Moreover, we had identified P-TEFb as a high-confidence interactor in our MS-based nuclear actin interactome analysis (20). Nevertheless, neither of these studies were able to establish whether the interaction between actin and P-TEFb is direct or not. To study this further, we first confirmed the interaction between these two proteins by using co-immunoprecipitation (co-IP) assay. Indeed, Flag-tagged Cdk9, the kinase subunit of P-TEFb, could be co-immunoprecipitated with HA-tagged actin but not with an empty vector control (Fig. 2*A*). To gain insight into the functional form of actin involved in this interaction, we then performed the experiment using actin mutants that are either predominantly monomeric (R62D, G13R) (26), favor polymerization (V159N) (26), or have mutations in the hydrophobic cleft, which is a commonly used site for actin-binding in many proteins (27). Rather surprisingly, all tested actin mutants were able to co-immunoprecipitate Cdk9, and the amount of Cdk9 detected in the IP sample seemed to correspond to the expression levels of Cdk9 construct in the input sample (Fig. 2*B*). Thus, this experiment did not provide further insights into the form of actin engaged with P-TEFb.

To study, if the binding between actin and P-TEFb is direct, we purified His-tagged Cdk9 from insect cells and actin from rabbit skeletal muscle. We then complexed the actin with Latrunculin B (LatB-actin) to perform a pulldown assay using His-Cdk9 as a bait under physiological salt conditions. LatB-actin was detected on beads coated with His-Cdk9 but not on beads with His-GST lysate (Fig. 2*C*). This assay

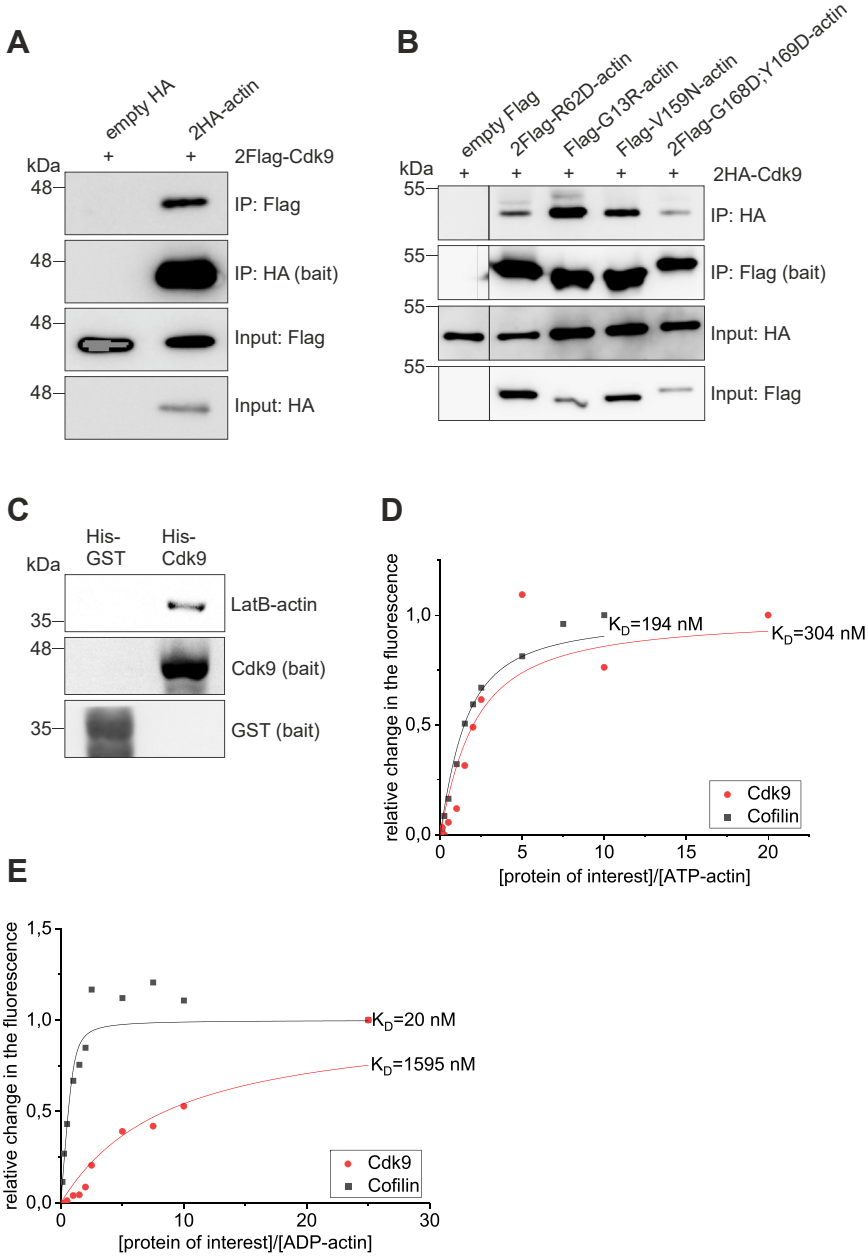


**Figure 2. Cdk9 binds directly to G-actin with a preference towards ATP-actin.** *A*, Western blot of co-immunoprecipitation (IP) experiment for 2Flag-Cdk9 (prey) and 2HA-actin (bait), using empty HA as a control. *B*, Western blot of co-IP experiment for 2HA-Cdk9 (prey) and indicated Flag/2Flag-tagged actin mutants (baits). Boxed areas cut from the same exposure to remove a construct with weak expression level and a lane with protein ladder. *C*, Western blot of pulldown assay of LatB-actin using His-Cdk9 as the bait and His-GST as a control bait. His-GST detected with His-HRP antibody, His-Cdk9 with Cdk9 antibody, and LatB-actin with Ac-40 actin antibody. *D*, NBD-actin monomer-binding assay showing increase in the fluorescence of 0.2 μM NBD-labeled ATP-Mg-G-actin under physiological ionic conditions over a range of concentrations of Cdk9 or known actin-binding protein cofilin. Symbols are data points and lines represent the exponentially fitted binding curves. N = 3, one example presented. Experimentally determined dissociation constants ($K_D$) calculated according to formula presented in materials and methods. Average $K_D$ of three assays is 375 ± 105 nM. *E*, NBD-actin monomer-binding assay as in (*A*), but using ADP-bound actin. LatB, Latrunculin B.

demonstrates a direct interaction between actin and the Cdk9 subunit of P-TEFb and also indicates that actin interacts with P-TEFb as a monomer.

Next, we used the NBD actin monomer-binding assay (Fig. 2, *D* and *E*) to characterize the interaction between actin and Cdk9 further. In this assay, lysine 372 of actin is labeled with NBD fluorophore and upon protein binding near this residue, fluorescence of NBD changes and the changes can be recorded (28). Actin is always bound to a nucleotide, ATP or ADP, and this is a critical factor that controls the polymerization process and interactions with other proteins. In addition, actin secondary structure is different between ATP- and ADP-bound forms (29). Many actin monomer-binding proteins, such as thymosin β4, profilin, and MIM prefer to bind ATP-actin (30–32), whereas cofilin and twinfilin have higher affinity towards ADP-actin (33, 34). With the NBD-actin assay, we observed that Cdk9 has a higher affinity towards ATP-actin (Fig. 2*D*) than ADP-actin (Fig. 2*E*). Average experimentally

defined dissociation constant ($K_D$) towards ATP-actin from three independent replicate experiments was 375 nM, but for ADP-actin, the $K_D$ could not be reliably defined, since the affinity was low. Cofilin was used as a control in our experiment and the $K_D$ values obtained experimentally here for cofilin binding match earlier results ($K_D$ = 20–100 nM towards ADP-actin) (33, 35, 36), verifying that the assay worked as expected. The NBD-actin assay thus further confirmed the direct interaction between actin monomers and Cdk9 and suggested that the interaction is sensitive to the nucleotide status of actin.

### *P-TEFb inhibits actin polymerization and the interaction is not dependent on the kinase activity of the complex*

To study how binding of P-TEFb affects actin, we next investigated actin polymerization with purified proteins by using pyrene actin polymerization assay (Fig. 3). In this assay, G-actin is labeled with fluorophore pyrene and buffer conditions are changed to favor actin polymerization, which can be measured by following the increase in pyrene fluorescence as filaments form (37). The P-TEFb complex used in these assays consisted of full-length Cdk9 and a C-terminally truncated version of Cyclin T1 (amino acids 1–272) containing the cyclin boxes only. This N-terminal domain of Cyclin T1 is widely used in both P-TEFb crystal structures (38) and *in vitro* biochemical experiments and demonstrated to activate Cdk9 (39).

Our results show that actin binding to P-TEFb (Fig. 3*A*) and to Cdk9 alone (Fig. 3*B*) inhibits actin polymerization in a concentration-dependent manner, likely by sequestering actin monomers. However, Cyclin T1 alone did not have any effect on actin polymerization (Fig. 3*C*), further confirming that

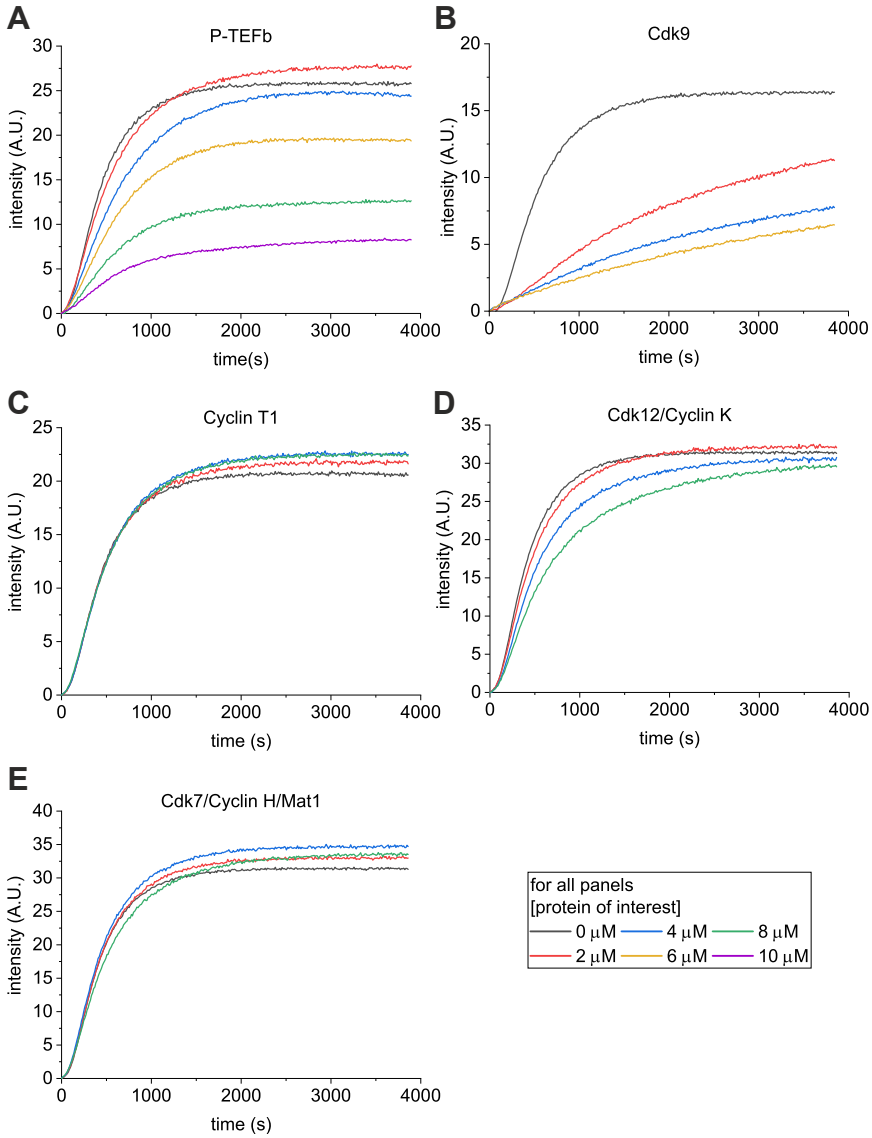


**Figure 3. Actin binds to P-TEFb *via* Cdk9 and the binding inhibits actin polymerization.** Pyrene actin polymerization assay with (*A*) P-TEFb, (*B*) Cdk9, (*C*) Cyclin T1, (*D*) Cdk12/Cyclin K, (*E*) Cdk7/Cyclin H/Mat1 added in increasing concentrations and actin polymerization monitored by increase in the pyrene fluorescence. Actin concentration 4 μM, except for in the experiment with Cdk9 where actin is 2.5 μM. An example graph from one assay is presented. P-TEFb, positive transcription elongation factor b.

Cdk9 is the binding partner for actin in the P-TEFb complex, as suggested already by our pulldown (Fig. 2*C*) and NBD-actin assays with Cdk9 (Fig. 2, *D* and *E*). To test, if the interaction is specific to P-TEFb, or a more general phenomenon for transcriptional Cdk/cyclin complexes, we tested purified Cdk12/Cyclin K and Cdk7/Cyclin H/Mat1 complexes in the pyrene actin assay (Fig. 3, *D* and *E*). Cdk7/Cyclin H/Mat1 did not influence actin polymerization (Fig. 3*E*), whereas Cdk12/Cyclin K modestly decreased actin polymerization (Fig. 3*D*), but the effect was clearly weaker than observed with P-TEFb.

Since actin binds to the P-TEFb complex *via* Cdk9, which is a kinase, we wondered whether the kinase activity of Cdk9 might be required for the interaction. To this end, we used Cdk9 inhibitors flavopiridol (40), NVP-2 (41), and iCdk9 (42) in the pyrene actin polymerization assay. None of the inhibitors alone influenced actin polymerization (Fig. 4*A*). Moreover, they also did not affect the ability of Cdk9 to inhibit actin polymerization (Fig. 4*A*), leading us to conclude that the kinase activity is not needed for Cdk9 binding to actin monomers. To explore this further, we took advantage of the strong binding of actin to the gene bodies of *hsp* genes upon their transcription activation with HS (Fig. 1, *C–E*) and performed ChIP-qPCR experiments on the HS-responding gene *Hsp70*. Interestingly, and in line with the biochemical experiments (Fig. 4*B*), inhibiting Cdk9 activity did not reduce actin binding to the *Hsp70* gene. Thus, kinase activity of Cdk9 is dispensable for both actin binding to P-TEFb and to actin recruitment to actively transcribing genes.

### *Actin does not release P-TEFb from the inhibitory complex and has a minor influence on its kinase activity*

In cells, most of P-TEFb is bound to an inhibitory 7SK snRNP complex that is needed to control P-TEFb kinase activity and consequently Pol II transcription (43). Apart from P-TEFb itself, our BioID assay for nuclear actin interacting proteins (20) identified P-TEFb regulators LARP7, BRD4, and DDX21 as hits. This led us to hypothesize that actin could control the association of P-TEFb with these factors or the release of P-TEFb from 7SK snRNP, since LARP7 is part of the inhibitory complex, binding P-TEFb to promote its incorporation into the 7SK snRNP and regulating the stability of the 7SK (43). To test this idea, we performed an *in vitro* P-TEFb release assay, in which 7SK snRNP from HeLa cells was immobilized with HEXIM1 antibody and purified muscle actin was added to the reaction as a possible release agent under conditions that keep actin monomeric. As expected, RNAse A released P-TEFb from the complex by degrading the 7SK RNA and thus reduced the amount of Cdk9 that was co-immunoprecipitated with HEXIM1 (Fig. 5*A*). However, actin, at two different concentrations, did not reduce Cdk9 in the HEXIM1 co-IP (Fig. 5*A*). Thus, at least under these *in vitro*

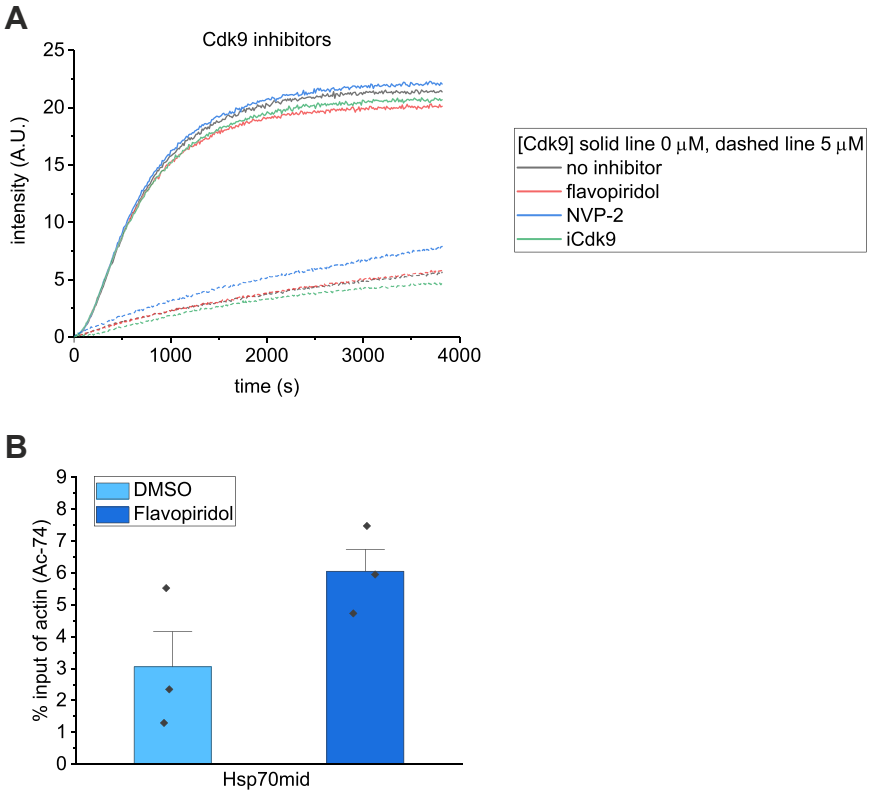


**Figure 4. Kinase activity of Cdk9 is not required for the interaction with actin or actin recruitment to *hsp* genes.** *A*, Pyrene actin polymerization assay with Cdk9 (0 and 5 μM) and Cdk9 inhibitors. Flavopiridol was 15 μM, NVP-2 5 μM, and iCdk9 10 μM. Actin concentration, 2.5 μM. *B*, chromatin immunoprecipitation with actin Ac-74 antibody followed by RT-qPCR (ChIP-qPCR) of *hsp70* middle region (Hsp70mid) in *Drosophila* S2R+ cells. Cells were treated with DMSO/Flavopiridol for 10 min, followed by 20 min heat shock and immediate fixing. N = 3, error bars ± 0.5 SD. Data presented as % of input enrichment. Statistical significance tested with two-sample *t* test, DMSO *versus* Flavopiridol $p = 0.130$.

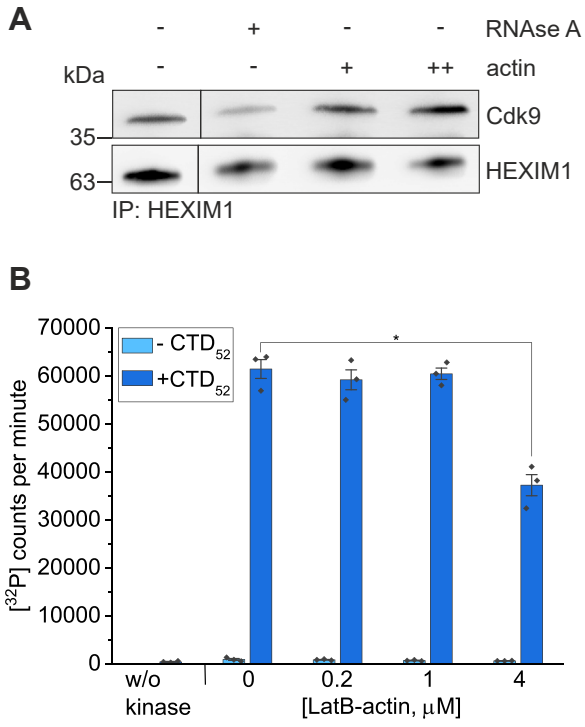


**Figure 5. *In vitro*, actin does not influence P-TEFb activity.** *A*, Western blot of P-TEFb release from HEXIM1 immunoprecipitation by RNAse A and increasing amounts of purified rabbit muscle actin (+0.5 μM and ++ 2 μM). P-TEFb probed with Cdk9 antibody and HEXIM1 with HEXIM1 antibody. In control sample (first lane), no release agent added. Boxed areas cut from the same exposure to remove an extra control sample. *B, in vitro* kinase assay assessing recombinant P-TEFb kinase activity for phosphorylation of $CTD_{52}$ substrate, and without the substrate as a control, with increasing LatB-actin amounts added to the reaction. Mean of triplicate measurements is presented, error bars ± 0.5 SD. *Statistically significant differences ($p < 0.05$) with a two-sample *t* test for samples with CTD substrate: 0 *versus* 0.2 μM actin $p = 0.532$, 0 *versus*. 1 μM actin $p = 0.730$, 0 *versus*. 4 μM actin $p = 0.002$. P-TEFb, positive transcription elongation factor b; LatB, Latrunculin B.

conditions, actin is not able to release P-TEFb from the inhibitory 7SK snRNP complex.

Another intriguing possibility is that actin could regulate Cdk9 kinase activity. The function of P-TEFb in releasing paused Pol II is based on Cdk9 phosphorylating the negative transcription elongation factors NELF and DSIF and serines at position 2 in the Pol II C-terminal domain (CTD) (44). We thus set out to do an *in vitro* kinase assay, in which the phosphorylation of a biologically relevant CTD substrate by P-TEFb in the absence and presence of LatB-actin was analyzed by monitoring radioactive ATP. Based on our results, actin does inhibit P-TEFb kinase activity but only at high concentrations (Fig. 5*B*). Significant effect was observed only when actin was used at 20 times higher concentration than P-TEFb, in line with the relative affinity measured for the Cdk9–actin interaction (Fig. 2*D*).

## Discussion

Actin was linked to RNA polymerase function already almost 40 years ago (24). Since then, several studies have demonstrated defects in transcription upon actin manipulations, whether with antibodies against actin (8, 12, 45), by altering nucleocytoplasmic shuttling of actin (2, 9, 13, 46, 47), by actin mutants that are either predominantly monomeric or filamentous (13–16, 46, 48, 49) or by using different actin-binding drugs (15, 45, 50, 51). Nevertheless, the molecular mechanisms by which actin affects RNA polymerase function have remained rather unclear. Here we use ChIP-seq and biochemical experiments with purified proteins to demonstrate that actin interacts with genes only upon their active transcription elongation and that actin binds directly to the Cdk9 kinase subunit of P-TEFb. Since P-TEFb plays an essential role in releasing paused Pol II to productive transcription elongation, we postulate that this interaction recruits actin to elongating polymerase to facilitate cotranscriptional processes.

Earlier studies on selected target genes had indicated variable chromatin-binding patterns for actin, with some studies finding actin only on promoter regions (7), some also on the gene coding regions (8, 19), and yet others also on intragenic sites (15). Our ChIP-seq studies in *Drosophila* ovaries revealed that actin binds to the promoter region of essentially all transcribed genes, but on highly expressed genes, it is also found on the gene bodies (9). Thus, the chromatin-binding pattern of actin depends on the expression level of the gene. Nevertheless, our earlier study was not able to discern the exact step of transcription, where actin plays a role. Here we took advantage of the fact that actin is strongly associated with *hsp* genes (Fig. 1, *C–E*), which have been widely used to dissect transcriptional mechanisms due to their robust activation upon HS. We found that actin associates with *hsp* genes only upon their active transcription elongation and is not significantly present on the genes in the absence of HS, although the RNA polymerase II can be readily detected as the paused polymerase (Fig. 1*C*). This indicated a functional role for actin specifically during the transcription elongation phase. However, we cannot exclude the possibility that actin could play a role also during other steps of transcription. For example, actin has also been linked to preinitiation complex assembly (12). It is possible that actin in this complex is not accessible to the antibody utilized here, warranting development of further reagents to study chromatin-binding properties of different forms of actin.

Actin has been demonstrated to copurify with all three RNA polymerases (7, 21–23). Nevertheless, it has not been detected as an integral polymerase subunit in any of the high-resolution structural studies that have recently been conducted, for example, using cryo-EM methods (52–54). Accordingly, we did not detect actin in the highly pure Pol II prep (Fig. S1*B*) and actin also did not bind to the purified Pol II in SEC experiments (Fig. S1*C*). In this latter case, as well as in some other experiments in this study (Figs. S1*C*, S2*C*, and S5*B*), we had to use actin complexed with LatB to overcome the limitation of actin polymerizing in physiological salt conditions. Even though LatB is a small molecule polymerization inhibitor, it can prevent interactions taking place at the exact same position on actin. Latrunculins bind near the nucleotide-binding cleft of actin (55, 56), which leaves the cleft between subdomains 1 and 3 where most actin-binding proteins bind free for interactions (57). It is, however, possible that LatB prevents the interaction between actin and Pol II. Another intriguing idea is that actin could interact with Pol II as a filament.

Indeed, Pol II clusters formed upon serum stimulation colocalize with nuclear actin filaments (13) and the links of nuclear myosins to Pol function have raised the idea of an acto-myosin motor operating during transcription (58). Unfortunately, we were not able to test this biochemically, since the purified Pol II cosedimented not only with actin filaments but also with microtubules (data not shown).

Earlier studies with co-IP techniques (16) and our own nuclear actin interactome analysis (20) had shown an interaction between actin and P-TEFb but not provided any biochemical details of this interaction. Here we significantly extend these studies by demonstrating that the interaction between actin and Cdk9, the kinase subunit of P-TEFb, is direct. Using purified proteins, the direct interaction was detected in a pulldown (Fig. 2*C*), pyrene actin polymerization (Fig. 3, *A* and *B*), and NBD-actin monomer binding (Fig. 2, *D* and *E*) assays, and the specificity of the binding demonstrated, for example, by using other transcription regulating Cdk/cyclin complexes (Fig. 3, *D* and *E*). Unfortunately, our attempts to gain further biochemical and structural information of the actin–P-TEFb complex proved unsuccessful. It might be that the interaction is very dynamic, which would also be supported with the relatively low affinity measured from the NBD-actin assays (Fig. 2, *D* and *E*) and the fact in the nuclear actin interactome analysis, P-TEFb was detected as a high-confidence interactor only with BioID, which detects also transient interactions, and not with affinity purification, which requires stable complex formation (20). In addition, the interaction might be electrostatic, which leads to practical problems when performing the assays with actin and P-TEFb. When purified, P-TEFb is usually kept in a high salt buffer to preserve its integrity and kinase activity, whereas actin polymerizes in high salt conditions. It therefore turned out to be difficult to find experimental conditions, where the salt concentration is high enough to maintain P-TEFb functional, and actin would be kept monomeric. Interestingly, also Cdk12/Cyclin K complex inhibited nuclear actin polymerization in the pyrene actin polymerization assays (Fig. 3*D*), but to lesser extent than Cdk9 or P-TEFb (Fig. 3, *A* and *B*), whereas Cdk7/Cyclin H/Mat1 did not influence pyrene actin polymerization (Fig. 3*E*). Of note, Cdk12/Cyclin K and Cdk9/Cyclin T1 share structural similarity, having open conformations (59), whereas Cdk7/Cyclin H form is more closed (60). In the future, it will be interesting to study, if actin may play a role in regulating Cdk12/Cyclin K complex, and cellular processes dependent on it.

It is tempting to speculate that the interaction between actin and P-TEFb plays a role in recruiting this complex to elongating Pol II. Indeed, Qi *et al.* (2011) (16) found that in nuclear extracts depleted of actin, Cdk9 was not recruited to immobilized transcriptional template, but the mechanism has remained unclear. In cells, most of P-TEFb is sequestered to the 7SK snRNP complex (43). Of note, one component of this complex, LARP7, was a hit in the BioID nuclear actin interactome assay (20). Thus, we speculated that actin could release P-TEFb from the 7SK snRNP and thereby promote its recruitment to Pol II. However, we did not obtain evidence of this in the P-TEFb release assay with increasing actin concentrations (Fig. 5*A*). Another intriguing possibility is that actin could influence P-TEFb by modulating the kinase activity of Cdk9. Qi *et al.* (2011) (16) performed an *in vitro* kinase assay using immunoprecipitation of different actin mutants overexpressed in HeLa nuclear extracts, after which CTD template and ATP were added to the reaction and the results are followed by Western blot. In this experimental setup, G-actin, but not predominantly filamentous actin, was found to stimulate serine 2 phosphorylation of Pol II CTD. We tested effects of actin to P-TEFb kinase activity using purified proteins to avoid possible effects of other proteins in nuclear extracts. We found that contrary to earlier results, high concentration of actin leads to less kinase activity (Fig. 5*B*). However, the effect was visible only at the highest actin concentration used. Therefore, it is not possible to conclude that in cells, actin would have a significant role in modulating the kinase activity of P-TEFb. It therefore seems that the stimulatory effect of actin on Cdk9 kinase activity detected earlier (16) is not direct but mediated by another protein that remains to be identified.

Intriguingly, the kinase activity of Cdk9 is not required for its interaction with actin, since the interaction persisted in the presence of different Cdk9 inhibitors (Fig. 4*A*). Moreover, actin accumulates to *Hsp70* gene upon heat shock even when flavopiridol is present (Fig. 4*B*). It has been reported that although flavopiridol reduces Pol II CTD Ser2 phosphorylation, it does not affect Pol II or P-TEFb density on genes (61). This indicates that the kinase activity is not needed for recruitment of P-TEFb to the gene. Hence, we currently favor a model, where the direct binding to Cdk9 recruits actin to the elongating Pol II. There, actin, through some additional binding partners, could stimulate Cdk9 activity as suggested earlier (16). This could explain why altering nuclear actin levels affects transcription elongation rate (20). In addition, actin could also recruit other nuclear complexes to facilitate cotranscriptional processes. For instance, earlier studies have demonstrated that actin interacts with several hnRNP proteins (18, 62, 63) and our nuclear actin interactome analysis identified numerous proteins implicated in RNA processing, including core components of the spliceosome (20). Detailed biochemical analysis of the underlying molecular interactions is needed to fully understand these tightly intertwined processes.

## Experimental procedures

### Plasmids and cloning

pEF-Flag-G13R-actin and pEF-Flag-V159N-actin plasmids were a gift from Richard Treisman (The Francis Crick Institute). 2HA-actin was cloned with restriction digestion. 2Flag-R62D-actin and 2Flag-G168D;Y169D-actin were cloned as described in (20). pENTR221-Cdk9 plasmid was purchased from the Genome Biology Unit, University of Helsinki (clone number 100003812) and used to generate 2HA-Cdk9, 2Flag-Cdk9, and Cdk9-pDest10 baculoviral expression construct using pDest10 backbone from Thermo Fisher Scientific. His-Cdk9 (fl)/GST-Cyclin T1 (1-272), GST-Cdk7(2-346)/Cyclin H(1-323)/Mat1(1-309) and GST-Cdk12 (714-1063)/GST-CycK (1-267) in pACEBac1 expression constructs are described in (64).

### Antibodies

The following antibodies were from Merck: Actin Ac-40 (A3853, 1:1000), Actin Ac-74 (A2228), HRP-conjugated poly-Histidine (His-HRP, A7058, 1:5000), HRP-conjugated anti-goat IgG (A9452, 1:5000), HRP-conjugated anti-Flag M2 (A8592, 1:5000), and HRP-conjugated anti-HA (HA-7) (H6533, 1:5000). Rpb1 antibody (664906, 1:500) was from BioLegend and HEXIM1 from Everest Biotech (EB06964, 1:2000). Cdk9 antibody (2316S, 1:1000) was from Cell Signaling Technology. Anti-RNA polymerase II CTD repeat YSPTSPS (phospho S5) antibody (ab5408) was from Abcam. Normal mouse IgG antibody (sc-2025) was from Santa Cruz Biotechnology. The following antibodies were from Thermo Fisher Scientific: HRP-conjugated anti-rabbit IgG (G-21234, 1:5000), HRP-conjugated anti-mouse IgG (G-21040, 1:5000).

### Cell lines

HeLa cells obtained from Marikki Laiho (University of Helsinki) were cultured in DMEM High Glucose w Sodium Pyruvate w/o L-glutamine medium (EuroClone) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), penicillin-streptomycin (Thermo Fisher Scientific), and GlutaMAX (Thermo Fisher Scientific) in 5% $CO_2$ incubator at +37 °C. *Drosophila* Schneider cell line (S2R+) was obtained from Jussi Taipale (University of Helsinki) and cultured in Schneider's *Drosophila* Medium (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific) and 1 × antibiotic-antimycotic solution (penicillin, streptomycin, Amphotericin B; Thermo Fisher Scientific) at +25 °C incubator.

### Rabbit skeletal muscle actin purification and LatB-actin preparation

Actin was purified from rabbit muscle acetone powder (Pel-Freez Biologicals) as described in (65). LatB-actin was prepared by incubating rabbit skeletal muscle actin with a tenfold molar excess of LatB (Merck) overnight at +4 °C. 20× initiation buffer (2 M NaCl, 60 mM $MgCl_2$, 10 mM ATP) was added to polymerize uncomplexed actin and the possible filaments were removed by centrifugation at 200,000*g* for 15 min at +4 °C.

### RNA polymerase II purification

Pol II was purified from HeLa nuclear pellets as described in (66). Briefly, the nuclear pellets were solubilized to 50 mM Tris pH 7.9, 5 mM $MgCl_2$, 0.5 mM EDTA, 25% glycerol and sonicated. An ammonium sulfate precipitation with a 42% cut was performed, the pellets were resuspended, dialyzed to 150 mM ammonium sulfate, and placed on a DEAE52 anion exchange column (Whatman). After washing, Pol II was eluted to 400 mM ammonium sulfate, the protein containing fractions were pooled, and dialyzed to a buffer with 200 mM ammonium sulfate. Next, Pol II was bound to protein G Sepharose Fast Flow beads (GE Healthcare) containing 8WG16 antibody against Rpb1; beads were washed with high salt and Pol II was eluted to a buffer with a tri-heptapeptide repeat of the CTD and concentrated.

### Recombinant protein expression and purification

#### His-Cdk9

His-Cdk9 was expressed in insect cells using the MultiBac expression system. Cdk9-pDest10 plasmid was transformed into DH10MultiBac cells; recombinant bacmids were prepared and the presence of correct construct was verified by PCR. The bacmid was transfected into Sf9 cells using Fugene HD transfection reagent (Promega). The obtained viral stock was amplified and used for protein expression. The expression of His-Cdk9 was done by infecting cells at the density of 2 × $10^6$ cells/ml with the baculoviral stock at a multiplicity of infection of 1. After 96 h, the cells were collected by centrifugation.

His-Cdk9 was purified by lysing the cell pellets in lysis buffer (25 mM Tris–HCl pH 8.0, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.2% Igepal) supplemented with cOmplete EDTA free protease inhibitor cocktail (Merck) and benzonase nuclease (Merck). Cell lysate was homogenized using EmulsiFlex-C3 (Avestin) with gauge pressure of 15,000 psi for 10 min. After homogenization, NaCl concentration of the lysate was increased to 200 mM and 20 mM imidazole was added. Cell extract was clarified by centrifugation at 66,000*g*, 30 min at +4 °C. Supernatant was applied to HisTrap HP 5 ml column (GE Healthcare) pre-equilibrated in 25 mM Tris–HCl pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 20 mM imidazole buffer, column was washed with high salt (1 M NaCl) buffer, and gradient elution using 500 mM imidazole was performed. Fractions containing protein of interest were analyzed by SDS-PAGE and Coomassie staining, pooled, and injected to Superdex 200 HiLoad 16/600 gel filtration column (GE Healthcare) equilibrated with 25 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 10% glycerol buffer. Fractions were collected; protein containing fractions were analyzed and protein was concentrated and stored.

#### P-TEFb, Cdk7/CycH/Mat1, Cdk12/CycK expression

Recombinant proteins were expressed in Sf9 insect cells, except for P-TEFb cyclin subunit Cyclin T1 that was expressed in *Escherichia coli* BL21(DE3). Cdk12/CycK were co-expressed with CAK1 from *Saccharomyces cerevisiae*. Expressions done as described in (64).

#### P-TEFb purification

His-Cdk9 cell pellet was resuspended in lysis buffer (50 mM Hepes pH 7.6, 500 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole) supplemented with 1 mM PMSF and 1 μg/ml DNAseI. Lysate was sonicated, cleared by centrifugation, and applied to 5 ml HisTrap column (GE Healthcare) pre-equilibrated in lysis buffer. Column was extensively washed with wash buffer (50 mM Hepes, 1 M NaCl, 5 mM β-mercaptoethanol) and protein was eluted with elution buffer (50 mM Hepes, 500 mM NaCl, 5 mM β-mercaptoethanol, 500 mM imidazole) by gradient elution. Protein containing fractions were pooled and processed to TEV protease cleavage (10 μg/ml) overnight to cleave His-tag.

GST-Cyclin T1 bacterial pellet was resuspended in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM β-mercaptoethanol) supplemented with 1 mM PMSF and 1 μg/ml DNAseI. Lysate was sonicated, cleared by centrifugation, and added to Pierce Glutathione Agarose beads (Thermo Fisher Scientific) pre-washed with lysis buffer. Protein was eluted with elution buffer (50 mM Hepes, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM reduced glutathione) and eluted protein was processed to TEV protease cleavage overnight to cleave GST-tag.

Next day, proteins were centrifuged at 10,000*g*, 10 min at +4 °C to remove possible precipitates and combined in 1:1 M ratio to reconstitute the P-TEFb complex. The protein mixture was then loaded to Superdex 200 HiLoad 16/600 column (GE Healthcare) equilibrated with SEC buffer (50 mM Hepes pH 7.6, 500 mM NaCl, 1 mM TCEP, 10% glycerol). Peak fractions containing equal amounts of Cdk9 and Cyclin T1 as monitored by SDS-PAGE were collected, concentrated, and stored.

*Cdk7/CycH/Mat1 purification*

Purification described in (64).

*Cdk12/CycK purification*

Purification described in (59).

### Gel filtration

LatB-actin as a control (20 μM, 10 μl) or LatB-actin mixed with Pol II (2 μM, 10 μl) for 1 h on rotation was run to Superose 6 Increase 3.2/300 column (GE Healthcare) connected to Pharmacia SMART chromatography system and equilibrated with G-actin buffer (5 mM Tris–HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT) supplemented with 100 mM NaCl. Run was done at 0.06 ml/min and 120 μl fractions were collected until one column volume had passed. 4× SDS-PAGE loading buffer was added to fractions and they were processed to SDS-PAGE and Western blotting using actin Ac-40 and Pol II Rpb1 antibodies.

### Pulldown assay with purified proteins

His-GST lysate (10 μl) and purified His-Cdk9 (1 μM) in total volume of 300 μl were added to 50 μl of Ni-NTA agarose (Qiagen) equilibrated with binding buffer 1 (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM DTT) and reactions were incubated for 2 h on rotation at +4 °C. Beads were washed once with binding buffer 1, three times with binding buffer 2 (25 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM DTT), and once with binding buffer 1. LatB-actin (3 μM in total volume of 300 μl) was added to the beads and samples were incubated for 4 h on rotation at +4 °C. After incubation, beads were washed three times with binding buffer 1 and bound proteins were eluted to 1× SDS-PAGE loading buffer and processed for SDS-PAGE and Western blotting using His-HRP, Cdk9, and actin Ac-40 antibodies.

### *In vitro* kinase assay

Radioactive kinase measurements were done as described previously (64). Recombinant, purified P-TEFb with full-length Cdk9 and short Cyclin T1 (1–272), 0.2 μM, was mixed with 0 to 4 μM LatB-actin concentrations in kinase buffer (50 mM Hepes, 34 mM KCl, 7 mM $MgCl_2$, 2.5 mM DTE, 5 mM β-glycerol phosphate, pH 7.6). 0.2 mM final ATP concentration with 0.45 mCi [32P]-γ-ATP/ml were added and reactions were pre-incubated for 5 min. Reactions were then started by the addition of $CTD_{52}$ substrate (50 μM) and incubated for 15 min at 30 °C in a shaking incubator at 300 rpm. Reactions were stopped by adding EDTA to a final concentration of 50 mM. Reaction mixtures were spotted onto filter sheets (Amersham Protran nitrocellulose membrane, GE Healthcare) and washed 3 × with PBS. Radioactive counts were assessed in a Beckman Liquid Scintillation Counter for 1 min. Measurements were conducted in triplicates and the mean ± 0.5 SD is presented.

### NBD-actin monomer binding assay

NBD-actin (ATP bound) was purchased from Cytoskeleton. To prepare ADP-actin, hexokinase beads were used (67). CNBr-activated Sepharose 4B (GE Healthcare) were let to swell in 1 mM HCl pH 3.0 for 30 min and washed. Hexokinase (Merck) was diluted to coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl), added to the beads, and incubated overnight at +4 °C. Next day, reactive groups were blocked with 0.1 M Tris–HCl pH 8.0 for 1 h; beads were washed with 30 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$ and following materials were added: ATP-bound NBD-actin, 2.5 mM glucose, 0.4 mM ADP and 20 mM Tris-HCl pH 8.0. Beads were incubated for 2 h at +4 °C, after which ADP-NBD-actin was collected.

Measurement was done as described in (68). Reactions containing 10 × initiation mix (10 mM $MgCl_2$, 1 M KCl), NBD-actin in final concentration of 0.2 μM, and increasing concentrations of protein of interest in G-buffer (2 mM Tris–HCl pH 8, 0.1 mM $CaCl_2$, 0.2 mM ATP or ADP, 0.1 mM DTT) were pipetted to a black 96-well plate and fluorescence was measured at 482/520 nm, 1 s per well.

From the obtained data, the normalized enhancement of fluorescence was determined by the equation

$$E = \frac{(F - F_0)}{(F_{max} - F_0)}$$

where

$$E = normalized\ enhancement\ of\ fluorescence$$

$$F = fluorescence$$

$$F_0 = blank\ fluorescence$$

$$F_{max} = maximum\ fluorescence$$

Data were analyzed and fitted using Origin 2022b and the following equation

$$E = \frac{1}{2}c + \frac{1}{2}z - \frac{1}{2}\sqrt{(c+z)^2 - 4z}$$

where z and c are determined in the two following equations

$$z = \frac{[protein]tot}{[actin]tot} \text{ and } c = 1 + \frac{K_d}{[actin]tot} \text{ where}$$

$$Kd = equilibrium\ dissociation\ constant$$

Relative fluorescence was presented as a function of [protein of interest]/[actin]. Curves were used to see if the binding saturates and efficiency of actin-binding was calculated.

### Pyrene actin polymerization assay

Pyrene actin (Cytoskeleton) and purified rabbit muscle actin were diluted to 20 μM in G-actin buffer (5 mM Tris–HCl pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT) and mixed to obtain 5% pyrene labeled actin. Mixture was centrifuged at 312,500*g* for 30 min at +4 °C to ensure completely monomeric actin at the starting point of measurement. Reactions were prepared by diluting protein of interest to G-buffer and adding 10 × KME buffer (5 mM Tris–HCl pH 7.5, 1 M KCl, 50 mM $MgCl_2$, 10 mM EGTA) that creates conditions for actin polymerization. For pyrene actin assay with Cdk9 inhibitors, the inhibitors were also added at this point. Finally, actin was added to the reaction, reaction was transferred immediately to a cuvette, and fluorescence measurement was started to catch the starting point of actin polymerization. Measurement was done with sampling period of 10 s, illumination 5 nm, detection 10 nm, wavelengths 365 nm/407 nm, total time 4000 s. Data are shown by plotting the measured absorbance as a function of time.

### Co-immunoprecipitation

HeLa cells were plated on 10 cm cell culture dishes at a density of $1 \times 10^6$ cells per plate. Next day, cells were cotransfected with HA and Flag-tagged constructs (3 + 3 μg) using JetPrime transfection reagent (Polyplus) according to the manufacturer's protocol. After 2 days of transfection, cells were resuspended to cold IP-buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Igepal, and cOmplete EDTA free protease inhibitor cocktail (Merck)) and lysate was cleared by centrifugation. Pre-washed anti-Flag M2 affinity gel (Merck) or EZview Red Anti-HA Affinity Gel (Merck), 25 μl of slurry per sample, was added to lysates and samples were incubated on rotation at +4 °C for 4 h. After incubation, samples were washed three times with IP buffer without Igepal and bound proteins were eluted with 1× SDS-PAGE loading buffer and processed for SDS-PAGE and Western blotting using HA-HRP and Flag-HRP antibodies.

### P-TEFb release assay

P-TEFb release assay was performed as described in (69). Briefly, whole cell extract from one confluent 15 cm plate of HeLa cells was prepared by lysing the cells to 0.5 ml of buffer C (20 mM Tris–HCl, 150 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.5% Igepal, pH 7.9) supplemented with cOmplete EDTA free protease inhibitor cocktail and incubating for 1 h on rotation at +4 °C. After incubation, lysate was cleared by centrifugation. To immobilize 7SK snRNP, 1:2 diluted whole cell extract in buffer C was added to Dynabeads Protein G (Thermo Fisher Scientific) preincubated with HEXIM1 antibody, using 2 μg antibody and 20 μl protein G solution per sample, and incubated on rotation at +4 °C for 4 h. After incubation, beads were washed once with buffer C containing 350 mM NaCl, once with buffer C, and once with G-buffer (5 mM Tris–HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT). For release reactions, either 5 μl PureLink RNAse A (Thermo Fisher Scientific) or actin in increasing concentrations in G-buffer was added to the beads containing immobilized 7SK snRNP; reactions were incubated on rotation at +4 °C for 2 h; beads were washed once with G-buffer and once with buffer C and 1× SDS-PAGE loading buffer was added to the beads to elute bound proteins. Samples were processed to SDS-PAGE and Western blotting using HEXIM1 antibody to see equal amounts of HEXIM1 and Cdk9 antibody to observe possible release from 7SK snRNP.

### ChIP

S2R+ cells were splitted in T75 culture flasks and for ChIP-qPCR treated with 300 nM flavopiridol (MedChemExpress) or equal volume of DMSO for 10 min. Heat shock was performed by placing sealed flasks into +37 °C water bath for 20 min. Samples were crosslinked with 1% formaldehyde for 10 min, and crosslinking was quenched with ice-cold 125 mM glycine on ice for 5 min. Chromatin fragments were obtained from cells lysed in RIPA buffer (10 mM Tris–HCl pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) by sonication with Diagenode Bioruptor, 30 s on and 30 s off with the highest power setting, for 15 cycles in case of ChIP-seq and for eight cycles in ChIP-qPCR. Cells from one T75 flask were used for each immunoprecipitation. Immunoprecipitation was carried out with 5 μg of antibody (IgG, actin Ac-74 or Pol II S5P) overnight at +4 °C and immunecomplexes were collected with 50 μl of protein A sepharose (GE Healthcare) at +4 °C for 2 h on rotation. Chromatin was eluted from washed beads in 1% SDS in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). DNA purification after reverse crosslinking was done with phenol/chloroform/isoamyl alcohol (Thermo Fisher Scientific) and DNA was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and two volumes of ethanol using glycogen as a carrier. ChIP from fly ovaries has been described in (9).

### ChIP-seq

ChIP libraries were prepared using NEBNext ChIP-seq DNA Sample Prep Master Mix Set for Illumina (NEB E6240) and NEBNext Multiplex Oligos for Illumina (Index Primers Set 1, NEB E7335) according to manufacturer's instructions. Sequencing was done with Illumina NextSeq500 at Biomedicum Functional Genomics Unit (FuGU). ChIP-seq was performed in

duplicates. ChIP-seq data sets were aligned using Bowtie2 (using Chipster software; (70)) to version dm6 of *Drosophila* genome with the default settings. Genome coverage files were generated using DeepTools with RPKM normalization. To analyze, visualize, and present ChIP-seq data, we used Integrative Genomics Viewer (IGV, (71)) and DeepTools.

## Data availability

S2R+ ChIP-seq datasets generated in this manuscript have been deposited to Gene Expression Omnibus under accession GSE239771. ChIP-seq files corresponding to *Drosophila* $w^{1118}$ ovaries were downloaded from GEO under accession number GSE116362.

### ChIP-qPCR

ChIP-qPCR was performed using SensiFAST SYBR No-ROX One-Step Kit (Meridian Bioscience) according to manufacturer's instructions using 1:10 diluted inputs and ChIP samples without diluting.

Primers for *Drosophila* Hsp70 middle region (Hsp70mid), 5′ to 3′ orientation:

Forward: CGAGATTGACGCACTGTTTG.

Reverse: CACGATGTCGTGGATCTGAC.

Percent of input calculated noting the dilution factor of input, using the equation

$$\%\ input = 2^{((Cq(IN) - Log\ 2(DF)) - Cq(IP))} * 100$$

### Statistical analyses

Statistical testing was performed using Origin 2022b. Normality was tested and when data conformed to a normal distribution, two-tailed two-sample *t* test was used. All statistical tests were done with the significance level of 0.05.

*Supporting information*—This article contains supporting information.

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*Abbreviations*—The abbreviations used are: ABP, actin-binding protein; co-IP, co-immunoprecipitation; CTD, C-terminal domain; HS, heat shock; Hsp, heat shock protein; LatB, Latrunculin B; MRTF, myocardin-related transcription factor; MS, mass spectrometry; P-TEFb, positive transcription elongation factor b; SEC, size-exclusion chromatography; SRF, serum response factor; TSS, transcription start site.

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