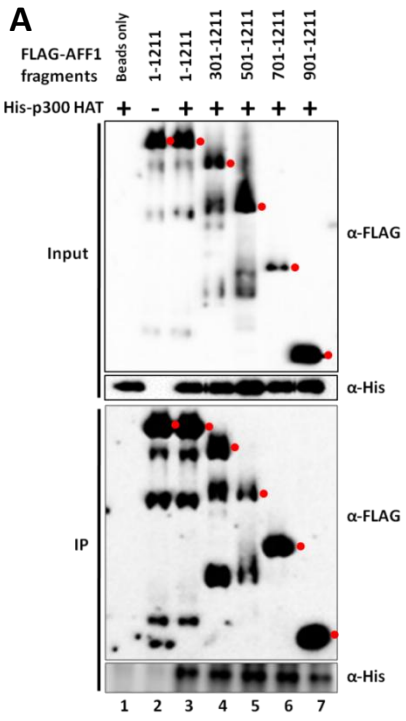
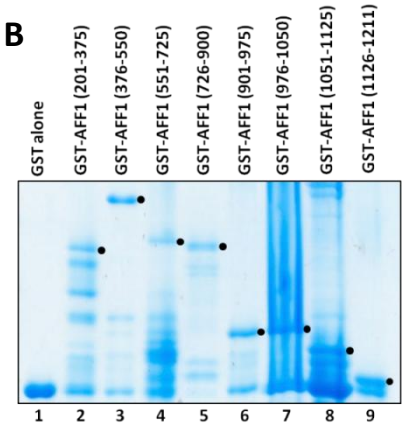
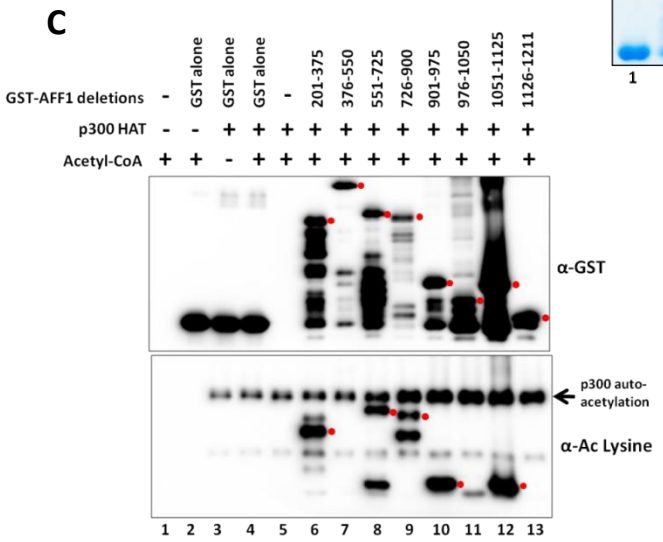
SI Appendix







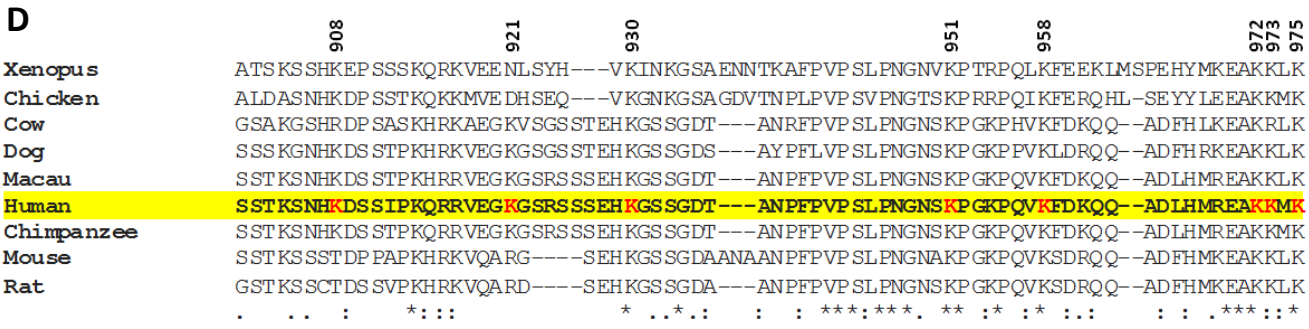


Figure S1: Purification and in vitro acetylation of recombinant AFF1 fragments

A. In vitro direct interaction assay showing interaction of purified several AFF1 fragments with p300 HAT domain.

B. Coomassie staining of indicated recombinant GST-AFF1 fragments that were expressed in bacteria and purified using glutathione agarose beads.

C. In vitro acetylation assays showing acetylation of purified AFF1 fragments (Fig. S1A) by p300. The top panel shows inputs for the acetylation reactions. The bottom panel shows acetylation of fragments as detected with a pan-acetyl lysine-specific antibody. The topmost bands (marked by red dots) indicate the target proteins in this experiment.

D. Multiple sequence alignment by Clustal Omega showing the presence of conserved AFF1 amino acids (from 901-975) across various species.

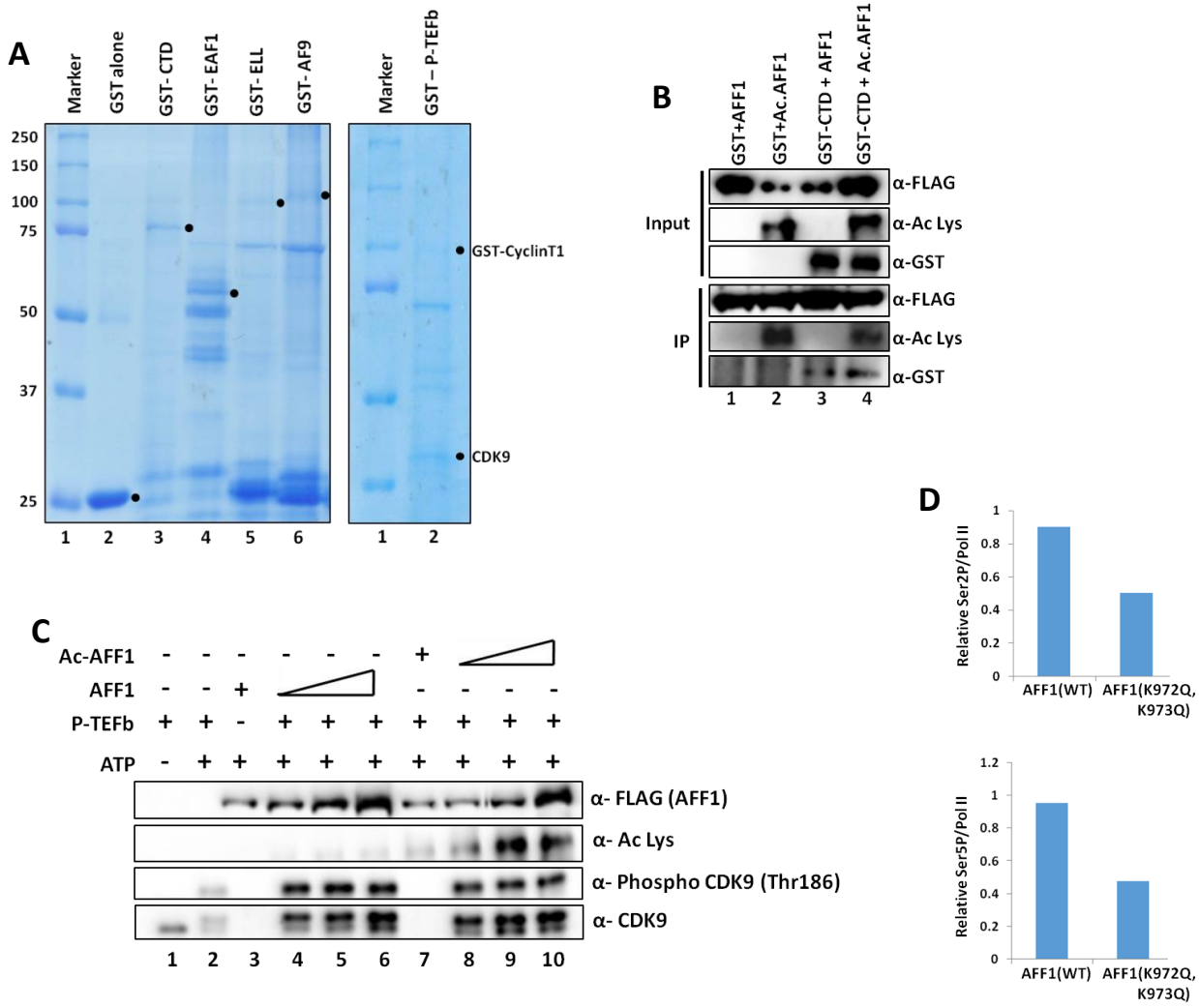


Figure S2: Effect of AFF1 acetylation on the direct interaction of AFF1 with the Pol II CTD, and Thr186 autophosphorylation activity of P-TEFb in vitro

A. Coomassie staining of recombinant proteins following expression through bacterial (GST-CTD, GST-EAF1, GST-ELL, and GST-AF9) or baculoviral (GST-P-TEFb) vectors and purifications using glutathione agarose beads or FLAG antibody (M2)-coated agarose beads. Filled black dots indicate the target protein band and others represent either degradation or non-specific proteins.

B. Effect of AFF1 acetylation on AFF1 interactions with GST-CTD. Analyses were carried out according to the scheme in Fig. 4D. Bound GST-CTD protein was monitored by immunoblot.

C. In vitro kinase assay with purified recombinant AFF1, acetylated AFF1, P-TEFb complex and GST-CTD (as substrate) to test the effect of AFF1 acetylation on autophosphorylation of Thr186 residue of CDK9 component of P-TEFb complex. An antibody specific to phospho-Thr186 CDK9 was used for detecting autophosphorylation of CDK9.

D. Quantified levels of phosphorylated Ser2 and Ser5 forms of Pol II CTD relative to total Pol II following over-expression of WT and (K972Q, K973Q) mutant forms of AFF1.

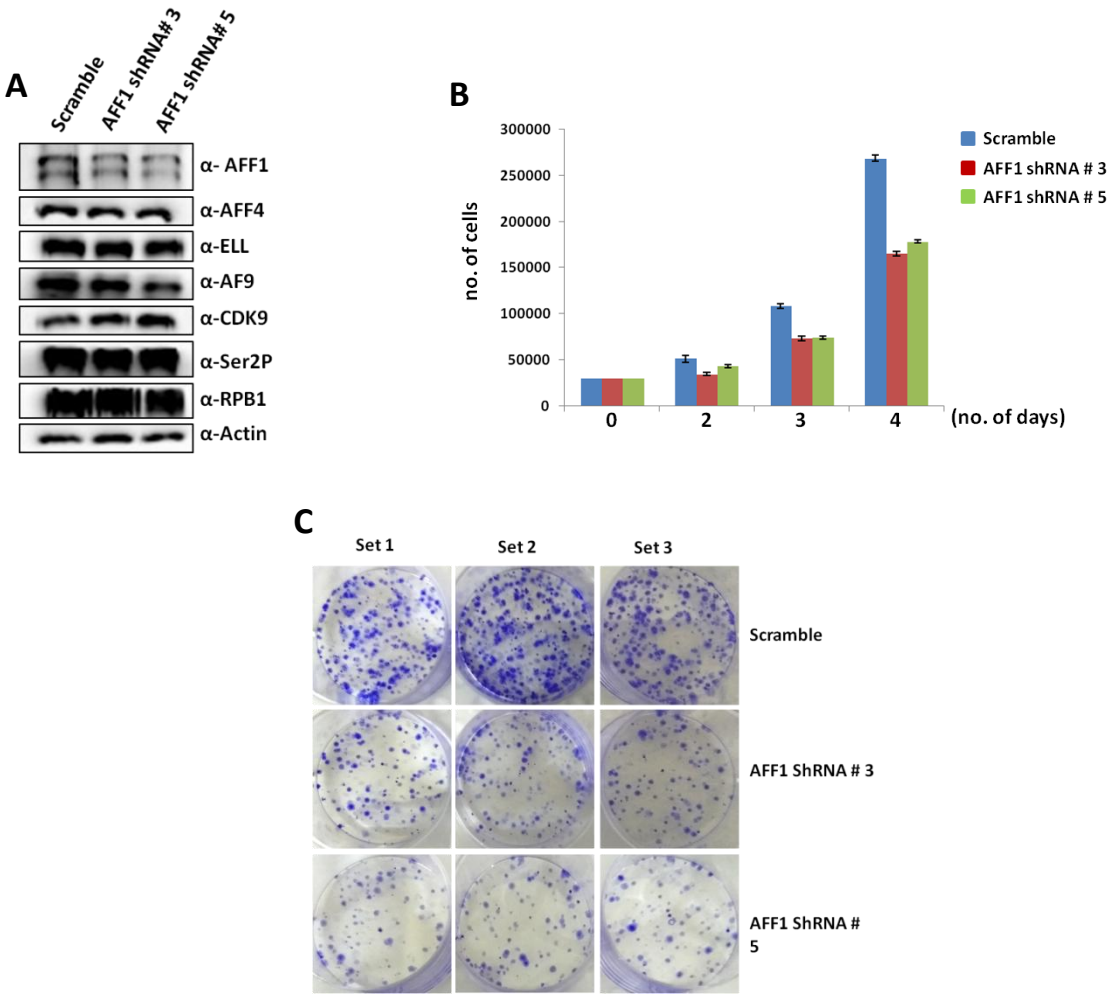


Figure S3: Effect of AFF1 knockdown on proliferation and colony formation potential of 293T cells

A. Immunoblot showing effect of AFF1 knockdown on expression of other AFF1-interacting proteins in 293T cells.

B. Cell proliferation assay showing effect of AFF1 knockdown on proliferation of 293T cells.

C. Colony formation assay showing effect of AFF1 knockdown on colony formation ability of 293T cells.

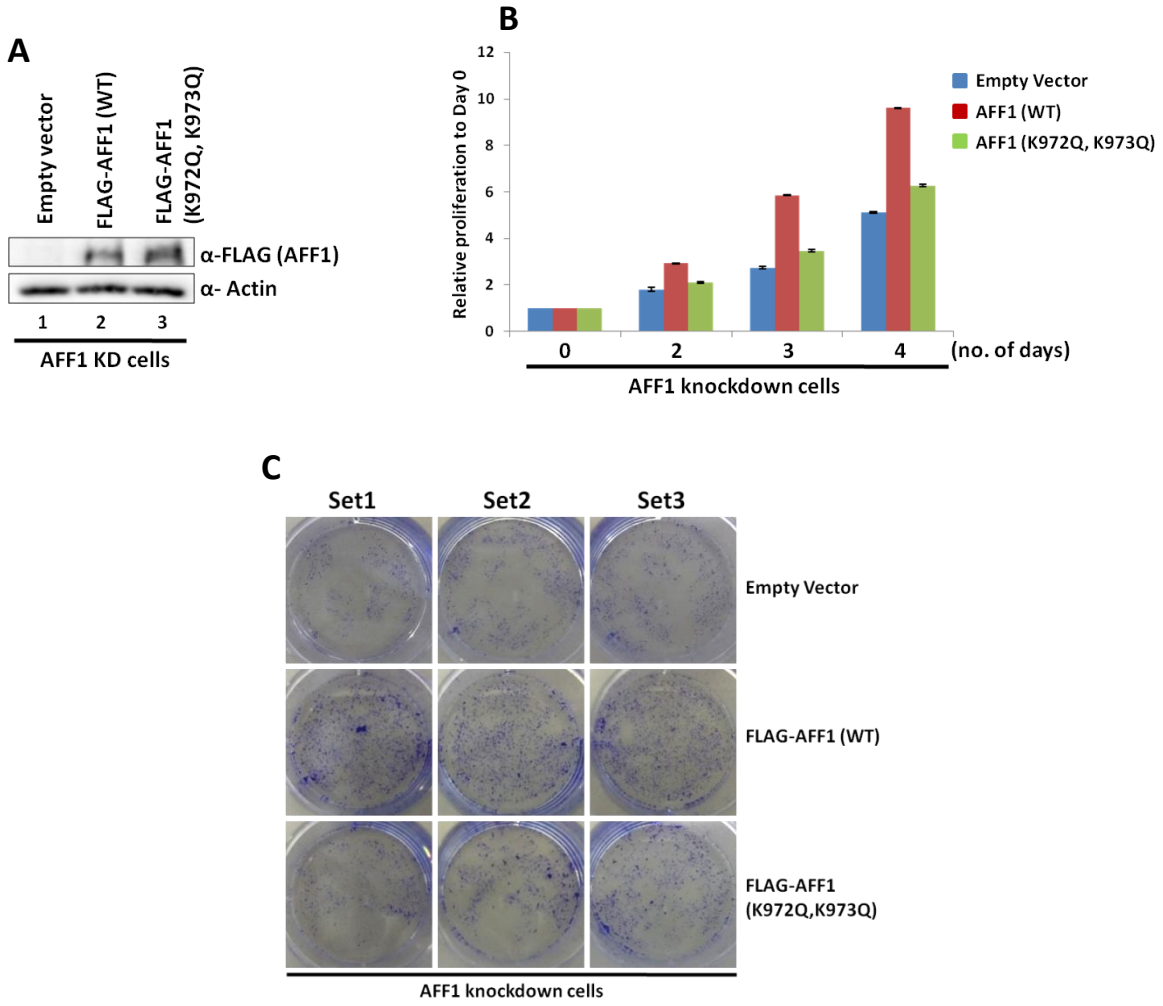


Figure S4: Effect of ectopic expression of WT AFF1 and AFF1 (K972Q, K973Q) mutant in AFF1 knockdown cells on overall proliferation and colony formation potential

A. Immunoblot showing expression of WT AFF1 and AFF1 (K972Q, K973Q) mutant in the AFF1-depleted 293T cells that were used for the proliferation and colony formation assays in Fig. S4B and S4C.

B. Cell proliferation assay showing effects of ectopic expression of WT AFF1 and AFF1 (K972Q, K973Q) mutant on overall proliferation in AFF1-depeleted 293T cells.

C. Colony formation assay showing effects of ectopic expression of WT AFF1 and AFF1 (K972Q, K973Q) mutant on overall colony formation in AFF1-depeleted 293T cells.

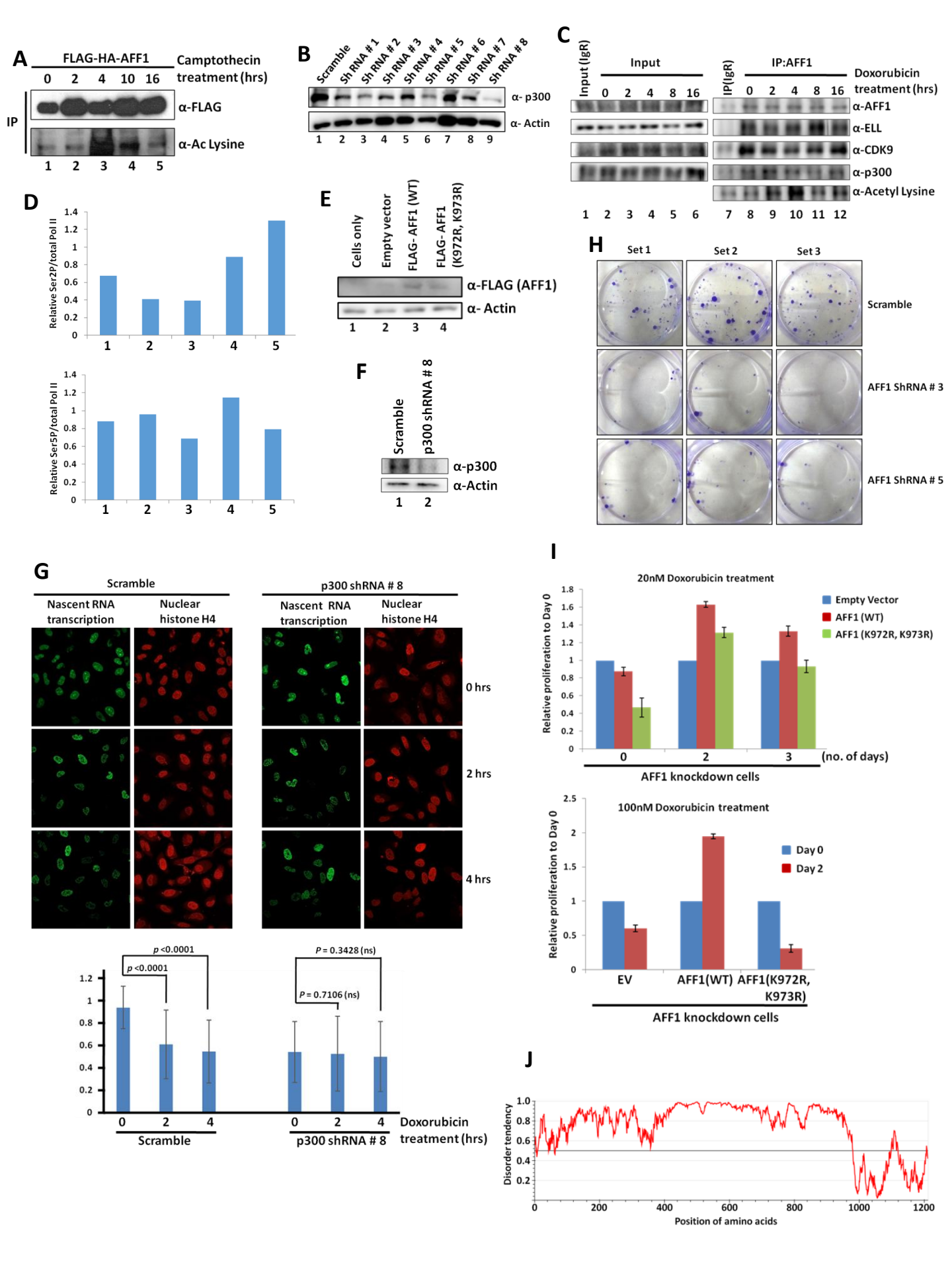

A
FLAG-HA-AFF1
0 2 4 10 16
Camptothecin treatment (hrs)
IP
α-FLAG
α-Ac Lysine
1 2 3 4 5
B
Scramble
Sh RNA # 1
Sh RNA # 2
Sh RNA # 3
Sh RNA # 4
Sh RNA # 5
Sh RNA # 6
Sh RNA # 7
Sh RNA # 8
α- p300
α- Actin
1 2 3 4 5 6 7 8 9
C
Input (IgR)
Input
0 2 4 8 16
IP(IgR)
IP:AFF1
0 2 4 8 16
Doxorubicin treatment (hrs)
α-AFF1
α-ELL
α-CDK9
α-p300
α-Acetyl Lysine
1 2 3 4 5 6 7 8 9 10 11 12
D
Relative Ser2P/total Pol II
Relative Ser5P/total Pol II
1 2 3 4 5
E
Cells only
Empty vector
FLAG- AFF1 (WT)
FLAG- AFF1 (K972R, K973R)
α-FLAG (AFF1)
α- Actin
1 2 3 4
F
Scramble
p300 shRNA # 8
α-p300
α-Actin
1 2
H
Set 1
Set 2
Set 3
Scramble
AFF1 ShRNA # 3
AFF1 ShRNA # 5
G
Scramble
p300 shRNA # 8
Nascent RNA transcription
Nuclear histone H4
0 hrs
2 hrs
4 hrs
p <0.0001
p <0.0001
P = 0.3428 (ns)
P = 0.7106 (ns)
0 2 4
Scramble
0 2 4
p300 shRNA # 8
Doxorubicin treatment (hrs)
I
20nM Doxorubicin treatment
Relative proliferation to Day 0
Empty Vector
AFF1 (WT)
AFF1 (K972R, K973R)
0 2 3 (no. of days)
AFF1 knockdown cells
100nM Doxorubicin treatment
Relative proliferation to Day 0
Day 0
Day 2
EV
AFF1(WT)
AFF1(K972R, K973R)
AFF1 knockdown cells
J
Disorder tendency
0 200 400 600 800 1000 1200
Position of amino acids

Figure S5: Dynamic acetylation of AFF1 regulates multiple events during exposure to genotoxic reagents

A. Immunoblot analysis showing dynamic AFF1 acetylation in 293T cells at various time points after camptothecin treatment.

B. Immunoblot analysis showing stable knockdown of p300 in 293T cells by several shRNA constructs.

C. Effect of doxorubicin on endogenous AFF1 interaction with p300 and its acetylation and factor association. 293T cells were treated for indicated times and anti-AFF1 immunoprecipitates were analyzed by immunoblot with indicated antibodies.

D. Quantified levels of phosphorylated Ser2 and Ser5 forms of Pol II CTD relative to total Pol II in 293T cells following doxorubicin treatment for various time points, as monitored by immunoblot analysis (as in Fig. 7E).

E. Immunoblot analysis showing expression of WT AFF1 and AFF1 (K972R, K973R) in the HeLa cells that were used for the analysis of nascent RNA synthesis in Fig. 7H.

F. Immunoblot analysis showing stable p300 knockdown in HeLa cells that were used for the analysis of nascent RNA synthesis in Fig. S5G.

G. Effect of doxorubicin on nascent RNA synthesis in control (Scramble) and p300 knockdown cells. Hela cells with control (Scramble) and p300 knockdown were treated for indicated times and imaged for 5-ethynyl uridine incorporation (left panels) or H4 (right panels). Quantitated data for nascent RNA synthesis(bottom panels) represent averages of 100 cells monitored for each time period. In all experiments, error bar represents mean ± SD, and p-values were calculated using one-tailed Student's t test, and ns denotes not significant.

H. Colony formation assay showing the effect of AFF1 knockdown on the colony formation ability of 293T cells after a 2 hr exposure to doxorubicin.

I. Cell proliferation assay showing effects of ectopic expression of WT AFF1 and AFF1 (K972R, K973R) mutant on overall proliferation of AFF1-depeleted 293T cells after prolonged exposure to low (20mM) (upper panel) and high (100mM) (lower panel) concentrations of doxorubicin for the indicated times.

J. Presence of intrinsically disordered regions within AFF1 as predicted by publicly available software IUPred2a (https://iupred2a.elte.hu/)

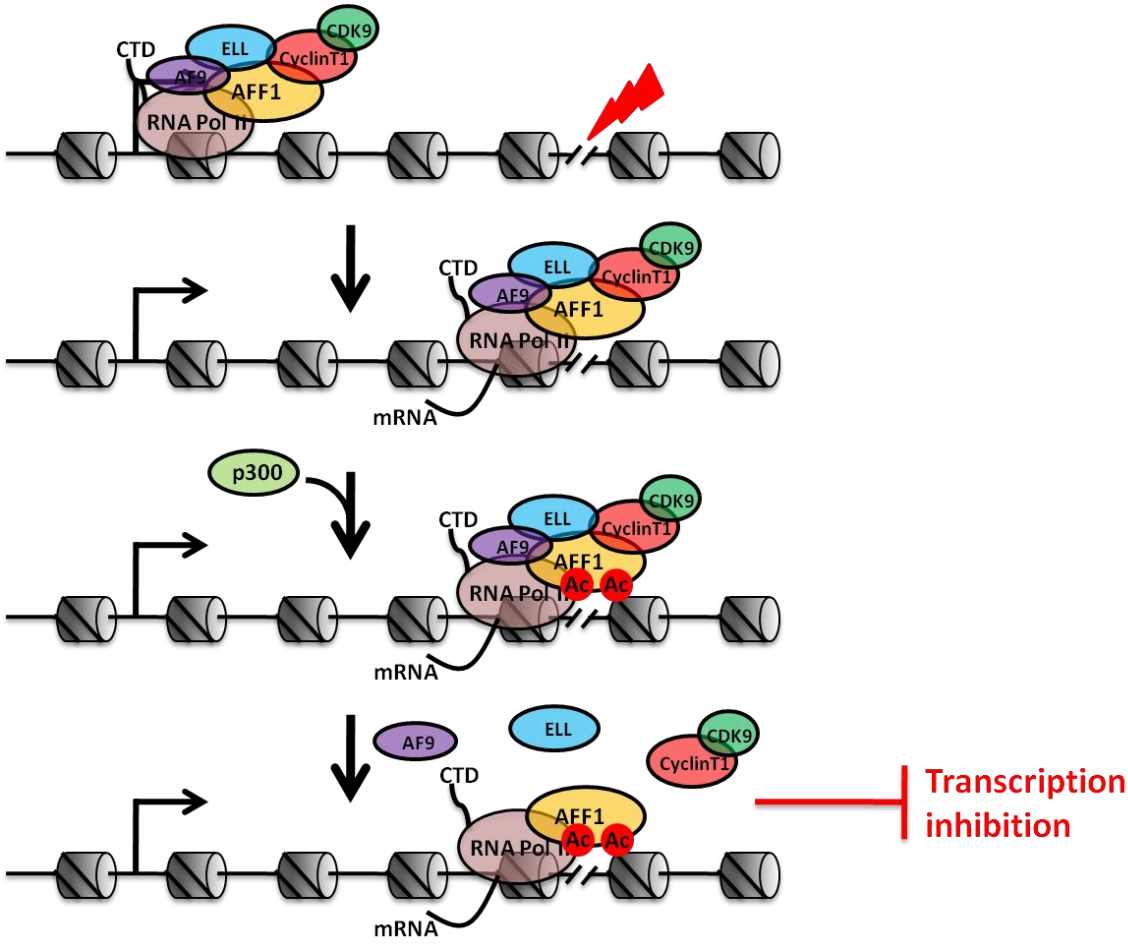


Figure S6: Model for the role of dynamic AFF1 acetylation in the regulation of transcription inhibition during genotoxic stress
Exposure of cells to genotoxic reagents leads to AFF1 acetylation by p300 and a consequent decrease in AFF1 interactions with cognate SEC members. Since AFF1 plays a scaffolding role in overall SEC assembly, a reduction in AFF1 interactions with other SEC components potentially leads to disassembly of a functional SEC complex and, thereby, temporally inhibits transcription.

Materials and Methods

Table S1: List of Plasmids used in this study.

Name of Plasmid	Description
M24	AFF1 cloned into FLAG-HA pCDNA5-FRT-TO vector
M532	p300 cloned into MYC pCDNA5-FRT-TO vector
M580	AFF4 cloned into FLAG-HA pCDNA5-FRT-TO vector
M902	AFF1 cloned into HA pCDNA5-FRT-TO vector
M378	pCI FLAG-PCAF plasmid construct from Addgene (Plasmid # 8941)
M794	pAdEasy Flag GCN5 (N-terminus) from Addgene (Plasmid # 14106)
M773	MOF cloned into HIS pCDNA5-FRT-TO vector
M694	AFF1 (1-1012) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M695	AFF1 (1-812) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M697	AFF1 (1-412) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M698	AFF1 (1-212) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M700	AFF1 (301-1211) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M702	AFF1 (701-1211) fragment cloned into FLAG-HA pCDNA5-FRT-TO vector
M47	AFF1 cloned into FLAG-pFASTBAC vector
M123	AFF1 (1-1012) fragment cloned into FLAG-pFASTBAC vector
M124	AFF1 (1-812) fragment cloned into FLAG-pFASTBAC vector
M126	AFF1 (1-412) fragment cloned into FLAG-pFASTBAC vector
M127	AFF1 (1-212) fragment cloned into FLAG-pFASTBAC vector
M128	AFF1 (101-1211) fragment cloned into FLAG-pFASTBAC vector
M129	AFF1 (301-1211) fragment cloned into FLAG-pFASTBAC vector
M130	AFF1 (501-1211) fragment cloned into FLAG-pFASTBAC vector
M131	AFF1 (701-1211) fragment cloned into FLAG-pFASTBAC vector
M132	AFF1 (901-1211) fragment cloned into FLAG-pFASTBAC vector
M686	P300 HAT domain (428-557) cloned into pET-HIS vector
M763	AFF1 (201-375) fragment cloned into pET-GST vector
M764	AFF1 (376-550) fragment cloned into pET-GST vector
M765	AFF1 (551-725) fragment cloned into pET-GST vector
M766	AFF1 (726-900) fragment cloned into pET-GST vector
M767	AFF1 (901-975) fragment cloned into pET-GST vector
M768	AFF1 (976-1050) fragment cloned into pET-GST vector
M769	AFF1 (1051-1125) fragment cloned into pET-GST vector
M831	AFF1 point mutation (K908A) in M767
M832	AFF1 point mutation (K921A, K930A) in M767
M833	AFF1 point mutation (K951A, K958A) in M767
M834	AFF1 point mutation (K972A, K973A, K975A) in M767
M835	AFF1 point mutation (K908A ,K972A, K973A, K975A) in M767
M1019	AFF1 point mutant (K972Q and K973Q) in M24
M1076	AFF1 point mutant (K972R and K973R) in M24
M56	pET-GST vector from Amersham
M434	EAF1 cloned into pET-GST vector
M502	Pol II CTD repeat cloned into pET-GST vector

M61	ELL cloned into pET-GST vector
M300	AF9 cloned into pET-GST vector
M653	CyclinT1 cloned into GST-pFASTBAC vector
M76	CDK9 cloned into pFASTBAC vector
M251	CDK9 cloned into FLAG-pFASTBAC vector
M399	CyclinT1 cloned into pFASTBAC vector

Table S2: List of primers used for RNA analysis

Primer name	Forward primer sequence	Reverse primer sequence
18srRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
KLHL13	GAATGTTACAATCCAAGAAC	GTAATTCCTCCTGAAATATAC
CENPH	TCAGAGAGGATAAAGATCATAC	AGGATCCTCTGCCCAATTG
WDR34	AGTATCTGTTTGCTGTGCGC	TCAAAACTGTGGGTTTCTGG
SBF1	GGCTTCACACCCGTCTTCC	GGACACATGGTGGTAGCCG
STIP1	GAGAAAATCCTGAAGGAGCAAG	ATGCTTCATGGCCTGGGGATA
PTPRS	AACACAGAAGTGCCCGCAC	GTGACGTGTGGGCCTTGGAG
PTPRF	CAATGCCAGCTTCCTGGATG	ATGATGGTGGAATTGTGCTC
RPTOR	TTGAGCATGCCGTCAATAATAC	TGACCGCTAAAGAACGGGTATA
CDK8	CAGCCTTATCAAGTATATGG	TGCATAGCCTGTTCTGAG
CCND1	TCTAAGATGAAGGAGACCAT	GGAAGTGTTCAATGAAATCG
CDKN1B	CGACGATTCTTCTACTCAA	TTACGTTTGACGTCTTCTG
CDKN2C	GGATTTGGAAGGACTGCG	GCTTGAAACTCCAGCAAAGTC

Table S3: List of primers used in ChIP analyses

Primer name	Forward primer sequence	Reverse primer sequence
CDKN1B TSS	GGTTTGTTGGCAGCAGTAC	GGAGATCCATTGGTTGCGG
CDKN1B +2kb	AGAGGTTCAGCTTGAGTCGG	GCAATTTCTACCAAACATTCCCC
CENPH TSS	TCCATTTAGTGGCGGGAAAAG	TTGCATCTGGGGCTGCTC
CENPH +2kb	ACCTGGCCTCAAATAAGATTTTG	CCAGTTTTGCCACACTATAAC
PTPRF TSS	TCCAGCTTCGGCTCCGGC	TCCACCTGGTCCCGCTCC
PTPRF +2kb	TTTATAGTTTCCTTTTCCAGCTG	AGAAAGGGAGGAAGGAAGGAAG
RPTOR TSS	GGCAATATGGCGTCCTCCTTG	GGACAATGTGGGCTTATTGGG
RPTOR +2kb	GGACAGAGTGAGACTGTGTC	GCCAAGTCAGTAGCTAACTGG
STIP1 TSS	CTCCCATATATCAGGGGCGG	GATTCCAATTGGGCGGAGGG
STIP1 +2kb	AGCTTGCGGTTATGAAAGGC	TCTTGGAGGTTGTTTTGTCC

Table S4: List of antibodies used

Name of factor	Source	Catalogue number
CyclinT1	Santa Cruz Biotechnology	sc-10750
CDK9	Santa Cruz Biotechnology and Cell Signaling Technology	sc-8338 and 2316

FLAG epitope tag	Sigma	F7425
GST	Santa Cruz Biotechnology	sc-53909
AFF1	Abcam	ab31812
AF9	Bethyl Lab	A300-596
Rpb1 (4H8 clone)	Cell Signaling Technology	2629
Phospho Rpb1 CTD (Ser2)	Cell Signaling Technology	13523
Phospho Rpb1 CTD (Ser5)	Cell Signaling Technology	13523
EAF2	Bethyl Lab	A302-503A
ELL	Bethyl Lab and Cell Signaling Technology	A301-645A, 14468
c-MYC (9E10)	Santa Cruz Biotechnology	sc-40
Acetyl Lysine	Cell Signaling Technology	9441
His tag	Santa Cruz Biotechnology	sc-8036
HA tag	Santa Cruz Biotechnology	sc-57592
ENL	Cell Signaling Technology	14893
EAF1	Santa Cruz Biotechnology	sc-373832
α- Phospho CDK9 Thr186	Cell Signaling Technology	2549
Histone 4	Cell Signaling Technology	
Beta Actin	Santa Cruz Biotechnology	sc47778

Cell Culture and Transfection

HEK293T and HeLa cells, used in this study, were grown in DMEM media supplemented with 10% FBS and 1% penicillin and streptomycin. All mammalian cell lines were grown in 37°C incubator with 5% CO_2. Transfections were performed using Lipofectamine 2000 (Thermo Fischer Scientific) following manufacturer's protocol. Cells were harvested at different time points as indicated. For immunoprecipitation analysis, cells were usually harvested at 48 hours post transfection, unless otherwise mentioned. Sf9 insect cells were grown at 26°C in Grace's insect media (HiMedia) supplemented with 10% FBS and gentamycin (7μg/ml).

Generation of Plasmid constructs

Full-length AFF1, p300, MOF, and AFF1-deletion constructs used in this study were cloned into 1x-FLAG and HA epitope tag-containing pCDNA5-FRT-TO vector for the purpose of target factor expression in mammalian cells. Full-length AFF1, CDK9, CylinT1, and AFF1-deletion constructs were also cloned into 1x-epitope tag-containing pFASTBac vector for subsequent expression of target factor in Sf9-cell-based baculovirus expression system. AFF1-deletion constructs were also cloned into GST-tag vector and p300 HAT domain was cloned into 6-His pET-11d vector for further purification from bacterial system . For the purpose of each cloning, appropriate restriction enzymes were used. Cloning details will be available upon request from the corresponding authors of this study. Point mutants of AFF1 were generated through Site-directed mutagenesis kit from Stratagene and confirmed by sequencing before being used in experiments.

Recombinant protein purification

For purification using bacterial expression system, EAF1, Pol II CTD, ELL, AF9, AFF1 deletions or mutant fragments, and p300 HAT domain were transformed in BL21 DE3 *E.Coli* cells. Subsequently, protein expression was induced by adding 1mM IPTG and further growing cells for 6hrs at 37°C. Cells were harvested and resuspended in BC300 buffer (20mM Tris-Cl pH 8, 300mM KCl, 2mM EDTA, 20% Glycerol) supplemented with 2mM PMSF, 0.7µl/ml β-mercaptoethanol and 0.1% NP40. Resuspended cells were sonicated for 5 minutes with a setting of 30 sec on and off pulse. Lysate was centrifuged at 12,000 rpm for 20 minutes at 4°C and cleared supernatant was collected and incubated with GST beads for 4 hours at 4°C. Beads were washed thrice with BC300 buffer. Bound proteins were eluted in 100mM Tris-Cl pH 8.0 containing 30mM L-Glutathione reduced (Sigma).

For purification of His-tagged p300 HAT domain, secondary culture was grown at 25°C for 8-10 hours before induction. Protein expression was induced by adding 1mM IPTG and further growing cells for 12hrs at 25°C. Cells were resuspended in lysis buffer (50mM Na_2HPO_4, 300mM NaCl, 10mM imidazole, 20% Glycerol) containing 2mM PMSF and 0.1% NP40. Beads were washed 3 times with washing buffer (50mM Na_2HPO_4, 300mM NaCl, 20mM imidazole, 20% Glycerol, and 0.1% NP40) and protein was eluted in elution buffer (50mM Na_2HPO_4, 300mM NaCl, 250mM imidazole, 20% Glycerol, and 0.1% NP40).

Recombinant epitope-tagged protein from insect cells were purified through their expression using Bac-to-Bac baculoviral expression system (Invitrogen). Sf9 cells were infected with FLAG-AFF1 and its deletion fragments viruses or co-infected with GST-CyclinT1 and CDK9 viruses for P-TEFb complex purification. Post 48 hours of infection, cells were harvested and lysed in BC300 buffer supplemented with protease inhibitor cocktail (Sigma) for 2hours at 4°C. After centrifugation, supernatant was collected and incubated with FLAG-M2 agarose beads (Sigma) for overnight at 4°C. Beads were washed extensively with BC300 buffer. Bound proteins were eluted in BC100 buffer (20mM Tris-Cl pH 8.0, 100mM KCl, 2mM EDTA, 20% Glycerol, and 0.1% NP40) containing FLAG peptide (250ng/µl).

Immunoprecipitation and Western Blotting

For testing AFF1 acetylation in vivo, mammalian cells transfected with AFF1 expressing plasmids were lysed in RIPA buffer (50mM Tris-Cl pH 8, 150mM NaCl, 1% Triton X-100, and 0.2% SDS) containing 2mM PMSF and 0.7µl/ml β-mercaptoethanol for 45 minutes at 4°C. Subsequently, lysates were spun down at 12,000 rpm for 10 minutes at 4°C. Salt concentration of the collected soup was raised to 1M by adding required amount of 3M NaCl and was incubated with FLAG-M2 agarose beads for overnight. Next day, beads were washed extensively with RIPA buffer. For elution, protein-bound beads were boiled with 1X SDS loading dye at 95°C for 10 minutes. Eluted samples were subsequently used for immunoblot analysis using pan-acetyl lysine-specific antibody.

For protein-protein interaction analysis, cells were lysed in BC150 buffer (20mM Tris-Cl pH 8, 150mM KCl, 2mM EDTA, 20% Glycerol) supplemented with protease inhibitor cocktail, 0.1% NP40, 2mM PMSF and 0.7µl/ml β-mercaptoethanol for 2hr at 4°C and centrifuged at 12,000 rpm for 10 minutes. IP and elution was done similarly as mentioned above. Depending upon the protein size, 8 or 10% SDS gel was used. Transfer was done in nitrocellulose membrane (BIO-RAD) using 15% methanol for 90 or 120 minutes based on the required protein size. Membranes were blocked in 5% milk and incubated in

required primary antibodies overnight at 4°C. Subsequently, blots were washed thrice with 1x PBS containing 0.1% Tween-20 and incubated in anti-rabbit (1:10,000) or anti-mouse (1:5000) secondary antibody for 1hr at room temperature. Washes were repeated again and finally blots were developed using X-ray film or azure biosystems C400 chemidoc.

Nuclear extract preparation

To prepare nuclear extract, cells were harvested in PBS and packed cell volume (PCV) was measured. Cells were swelled by resuspending in 2X PCV of NE1 buffer (10mM Tris-Cl pH 7.3, 1.5mM $MgCl_2$, 10mM NaCl) containing 0.7µl/ml β-mercaptoethanol and incubated for 15 minutes on ice. Using 23 gauge needle, swollen cells were passaged through it for 8 times. Subsequently, cells were spun down at 5000 rpm for 10 minutes at 4^{o}C to obtain the nuclear pellet. The nuclear pellet volume (NPV) was measured and resuspended in 2X NPV of NE2 buffer (20mM Tris-Cl pH7.3, 1.5mM MgCl2, 20mM NaCl, 0.2mM EDTA, and 25% glycerol) containing protease inhibitor cocktail and 0.7 µl/ml β-mercaptoethanol. Subsequently, 1X NPV of NE3 buffer (20mM Tris-Cl pH7.3, 1.5mM MgCl2, 1.2M NaCl, 0.2mM EDTA, 25% glycerol) supplemented with protease inhibitor cocktail and 0.7µl/ml β-mercaptoethanol was added for extraction of nuclear proteins through mild vortexing. Samples were incubated on ice for 45 minutes with intermittent vortexing every after 3 minutes. To pellet nuclear debris, samples were centrifuged at 12,000 rpm for 20 minutes at 4°C. Finally, supernatant was collected as nuclear fraction for subsequent usage for IP and western blotting analyses.

Immunoprecipitation of endogenous proteins

Nuclear extract from HEK293T cells was prepared for setting up the endogenous IP. Prepared nuclear extract was pre-cleared with protein-A agarose beads (Invitrogen) for 1hr at 4°C. In a parallel setting, Protein G magnetic beads (BIO-RAD) were blocked in BC150 buffer containing 1% BSA(Sigma) and 0.1% NP40 for 1hr at 4°C. Blocked magnetic beads were washed thrice with BC150 buffer containing 0.1% NP40 and then incubated at 4°C for 4 hours with BC150 buffer containing 2µg of desired primary antibody. Pre-cleared nuclear extract was added to the primary antibody-bound magnetic beads and incubated overnight at 4°C. Beads were washed thrice with BC150 buffer containing 0.1% NP40. For protein elution, beads were boiled with 1X SDS loading dye at 95°C for 10 minutes. Endogenous protein association with the target factor was analyzed through western blotting using appropriate antibody as mentioned in the figures.

In vitro acetylation assay

Full-length AFF1 and its deletion or mutant fragments were either purified from Sf9 or bacterial cells, were used as substrate in the acetylation assay. Functionally-active p300 HAT domain was purified from bacterial cells as mentioned above. Reactions were carried out at 30^{o}C for 2 hours in a reaction containing 1X acetylation buffer (15mM Tris-Cl pH8.0, 0.25mM EDTA pH8.0, 0.05% Tween20, 2.5mM DTT, 5% glycerol, 500µM Acetyl-CoA). Acetylation of target proteins were analyzed by western blot using pan acetyl lysine-specific antibody.

Mass spectrometry of AFF1 peptide

Synthetic 9-mer AFF1 peptides (wild type and mutants, as indicated) were commercially purchased and subjected to in vitro acetylation for overnight using the protocol as mentioned above. Next, equal reaction volume of chilled 40% TCA was added to the acetylation reaction and after 10 minutes of incubation on ice, tubes were centrifuged at 14,000 rpm for 5 minutes to pellet down the peptides. Subsequently, pellet was washed twice with chilled acetone and air dried for 10 min at RT. Air-dried pellet was dissolved in 0.2% TFA and proceeded for mass spectrometry analysis.

In vitro Kinase assay

For the purpose of in vitro kinase assay functionally-active P-TEFb complex was purified through their expression in Sf9 cells. Purified recombinant GST-CTD through their expression in bacterial system was used as a substrate. Reactions were carried out at 30°C for 2 hours containing 1X kinase buffer (50mM Tris-Cl pH8.0, 10mM $MgCl_2$, 5mM DTT, 500µM ATP). Phosphorylation of GST-CTD substrate at Ser2 and Ser5 positions were analyzed by western blot analysis using phospho-Ser2 and -Ser5-specific antibodies.

In vitro binding assay

For in vitro binding assay, bead bound proteins were incubated with the purified protein (as indicated) in BC100 buffer containing 200ng/µl BSA and 0.07% NP40 at 4°C for overnight. Next day, factor bound beads were washed extensively with BC100 buffer containing 0.07% NP40. Bead-bound proteins were eluted in 1X SDS loading dye by heating at 95^{o}C for 10 minutes. Interacting proteins were identified through western blot analysis using appropriate antibodies as indicated.

Generation of stable Knockdown cells

Target shRNA sequences were cloned into lentiviral pLKO.1 puro vector (Addgene). Virus particles were generated through co-transfection of 250ng target shRNAs, 750ng of psPAX2 (packaging plasmid) and 500ng of pMD2.G plasmid (envelope plasmid) in HEK293T cells. Virus particles were collected post 72 hours of transfection. For generating stable knockdown, cells were transduced with virus particles in presence of 8µg/ml polybrene in a 6 well plate. Post 24 hours of transduction, cells were selected with puromycin (3µg/ml) containing complete DMEM media. Knockdown efficiency in puromycin positive cells for the target factor was confirmed through western blotting using factor-specific antibodies.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from knockdown or transfected cells of 6-well plate using TRIzol Reagent (Invitrogen) following manufacturer's protocol. 500ng of the total RNA was used for cDNA synthesis using verso cDNA synthesis kit (Thermo Scientific) following manufacturer's protocol. cDNA was diluted 25X for subsequent usage in qRT-PCR analysis using BIO-RAD CFX96™ Real-Time-System. iTaq Universal SYBR Green Supermix (BIO-RAD) and primers specific for the target genes were used for qRT-PCR analyses. Signal of target gene expression was normalized to 18S rRNA internal control.

ChIP assay

For ChIP assays, protocol as mentioned earlier (1, 2) was mostly followed with slight modifications. Target cells were cross-linked with 1% formaldehyde (Sigma) for 10 minutes and subsequently, fixation was stopped by addition of 125mM glycine (Sigma) for 5 minutes at room temperature. Cells were harvested in cold PBS and spun down at 3000 rpm for 10 minutes. Cells were allowed to swell in ice cold lysis buffer (0.5% NP-40, 1% Triton X-100, 150mM NaCl, 20mM Tris pH 7.5, 2mM EDTA) supplemented with protease inhibitor cocktail and incubated on ice for 30 minutes. Using 23gauge needle, swollen cells were passed through for 8 times. The lysed cells were spun down at 5000 rpm for 10 minutes at 4°C to obtain nuclear pellet. The nuclear pellet was resuspended in shearing buffer (1% SDS, 50mM Tris pH 8.0, 10mM EDTA) containing freshly added protease inhibitor cocktail and were subsequently sonicated using Bioruptor™ UCD-200 (Diagenode) sonicator for 25 minutes with a setting of 30sec pulse on and off. Sonicated samples were centrifuged at 12,000 rpm. Cleared supernatant was further diluted 10X in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.1mM EDTA, 20mM Tris-Cl (pH 8.0) and 167mM NaCl). Diluted extracts were initially pre-cleared with IgG for 1 hr and finally with protein G magnetic beads for further 1 hr at 4°C. Immunoprecipitation was carried out with the cleared lysate for overnight at 4°C using 2µg of desired target antibodies for approximately 65µg of sonicated DNA. Simultaneously, protein G magnetic beads were blocked by incubating with dilution buffer containing salmon sperm DNA (4µg/µl) for overnight at 4°C. Next day, blocked beads were added to the immunoprecipitated samples and were incubated further for 2hr at 4°C. Complex bound protein G beads were washed several times initially with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH 8.0, 150mM NaCl, and freshly added Protease inhibitor cocktail) followed by high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH 8.0, 500mM NaCl, and freshly added Protease inhibitor cocktail) followed by lithium chloride buffer (0.5M LiCl, 1% NP-40, 1% deoxycholate, 20mM Tris-Cl pH8.0,1mM EDTA and freshly added Protease inhibitor cocktail) and finally twice with TE buffer (10mM Tris-Cl pH8.0,1mM EDTA). The immunoprecipitated DNA was eluted using elution buffer (1% SDS, 0.1M $NaHCO_3$) at room temperature. Bound DNA was reverse cross-linked using 200mM NaCl through incubation at 65°C for overnight. Reverse cross-linked samples were treated with proteinase K (Sigma) by incubating at 45°C for 45 minutes. Immunoprecipitated DNA was eluted using PCR purification kit (QIAGEN). Eluted DNA was directly used for qRT-PCR analysis for checking target factor occupancy at the different region of the indicated gene using specific primers as indicated.

Immunofluorescence

HeLa cells were seeded on cover slips in 12-well plate and Click-iT™ RNA Alexa Fluor™ 488 Imaging Kit (Invitrogen) was used following manufacturer's protocol for detecting nascent transcription within the cells through immunofluorescence. After 30 hours of transfection, doxorubicin (500nM) treatment was carried out as mentioned in the figure. Target cells were treated with 0.3mM 5-ethynyl uridine (EU, provided in the kit) for 15 min for incorporation of EU to newly synthesized RNA. Cells were fixed with 4% paraformaldehyde (Sigma) for 15 minutes at room temperature and were subsequently washed with PBS. Fixed cells were subsequently permeabilized by treating with 0.5% Triton X-100 for 15 min. These cells were then blocked for 15 min by incubating in 1% BSA. Immediately after blocking, cells were washed with 1X PBS to remove additional blocking reagents. The blocked cells were incubated in Click-iTreaction cocktail for 30 minutes at room temperature and were subsequently washed with rinse

buffer. Cells were further incubated in H4 primary antibody (1:1000 in PBST) overnight at 4°C. Next day, cells were washed thrice with PBS and incubated in anti-rabbit Alexa Fluor 594 (1:500 in PBST) at 4°C. Cells were subsequently washed thrice with PBS. For DNA staining, the cells were incubated with Hoechst. The stained cells were proceeded for imaging with LSM 800 (ZEISS) microscope. Nascent RNA synthesis was calculated using Image-J software and were normalized with signal from Histone H4 as obtained. Signal from 100 cells were averaged for obtaining data about nascent RNA synthesis as shown in figures.

Colony formation assay

For colony formation assay, 1.5 x 10^4AFF1 knockdown cells were seeded in 6 well plate for the purpose of colony formation. Approximately, after 7-10 days of growth, cells were fixed with methanol: acetic acid (3:1) solution for 10 minutes at room temperature. Subsequently, colonies were stained with 0.5% crystal violet (in methanol) for 15 minutes. Colonies were washed several times with water to remove the excessive stain. Similarly, knockdown cells treated with 100nM doxorubicin for 2 hours, were also proceeded for colony formation assay following same method as described above.

Cell proliferation assay

For cell proliferation assay, 6 × 10^4 AFF1 knockdown cells were seeded in a 24 well plate. Cell numbers were counted on 2^{nd}, 3^{rd}, and 4^{th} day after seeding by using hemocytometer. Similarly, AFF1 knockdown cells in which wild type and mutant AFF1(K972R,K973R) expressions have been restored, were treated with 100nM doxorubicin for 2 hours and proceeded for cell proliferation assay following same protocol as described above. Post 48 hours of transfection in HEK293T cells with AFF1 wild type and AFF1 acetylation defective mutant, cells were treated with doxorubicin (20nM and 100nM) and were incubated for prolonged period of time until counting.

Fractionation of cytoplasmic and nuclear proteins

For the purpose of fractionation, cells were transfected with full length AFF1 and its deletion constructs. Post 48 hours of transfection, cells were harvested in PBS. Cell pellet was resuspended in cytoplasmic extraction buffer (10mM Tris-Cl pH8.0, 0.1mM EDTA pH8.0, 0.1% Triton X-100 , 50mM NaCl) supplemented with protease inhibitor cocktail. Lysis was carried out by rotating at 4^{o}C for 15 minutes. The lysed cells were centrifuged at 5000 rpm for 10 minutes to pellet the nucleus. Supernatant was collected as cytoplasmic fraction and re-centrifuged at 12,000 rpm for 10 minutes for removing any nuclear protein contamination. Nuclear pellet was washed thrice with washing buffer (10mM Tris-Cl pH8.0, 0.1mM EDTA pH8.0, 10mM KCl). 5X SDS loading dye was added to the cytoplasmic soup and 1X SDS loading dye was added to the nuclear pellet and was heated at 95^{o}C for 10 minutes before loading onto SDS-PAGE gel. Nuclear and cytoplasmic localization of the fragments were analyzed through western blotting keeping appropriate marker as a control as mentioned in the figures.

References:

1. Ghosh K*, et al.* (2018) Positive Regulation of Transcription by Human ZMYND8 through Its Association with P-TEFb Complex. *Cell Rep* 24(8):2141-2154 e2146.
2. Yadav D, Ghosh K, Basu S, Roeder RG, & Biswas D (2019) Multivalent Role of Human TFIID in Recruiting Elongation Components at the Promoter-Proximal Region for Transcriptional Control. *Cell Rep* 26(5):1303-1317 e1307.