Molecular Cell, Volume *52*

Supplemental Information

Acetylation of RNA Polymerase II Regulates Growth-Factor-Induced Gene Transcription in Mammalian Cells

Sebastian Schröder, Eva Herker, Friederike Itzen, Daniel He, Sean Thomas, Daniel A. Gilchrist, Katrin Kaehlcke, Sungyoo Cho, Katherine S. Pollard, John A. Capra, Martina Schnölzer, Philip A. Cole, Matthias Geyer, Benoit G. Bruneau, Karen Adelman, and Melanie Ott

SUPPLEMENTAL INFORMATION

INVENTORY OF SUPPLEMENTAL INFORMATION

Supplemental Data

Supplemental Experimental Procedures

Supplemental References

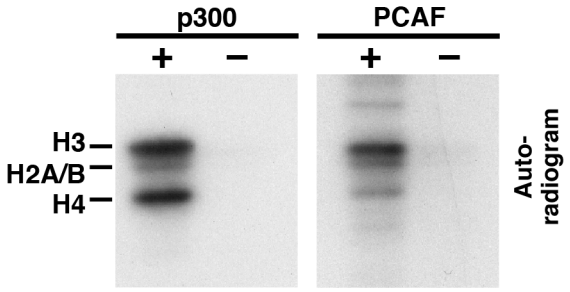

Figure S1: Purified p300 and PCAF acetylate histones, related to Figure 1
In vitro acetylation of purified histones with recombinant p300 or PCAF. Both enzymes acetylate purified histones efficiently.

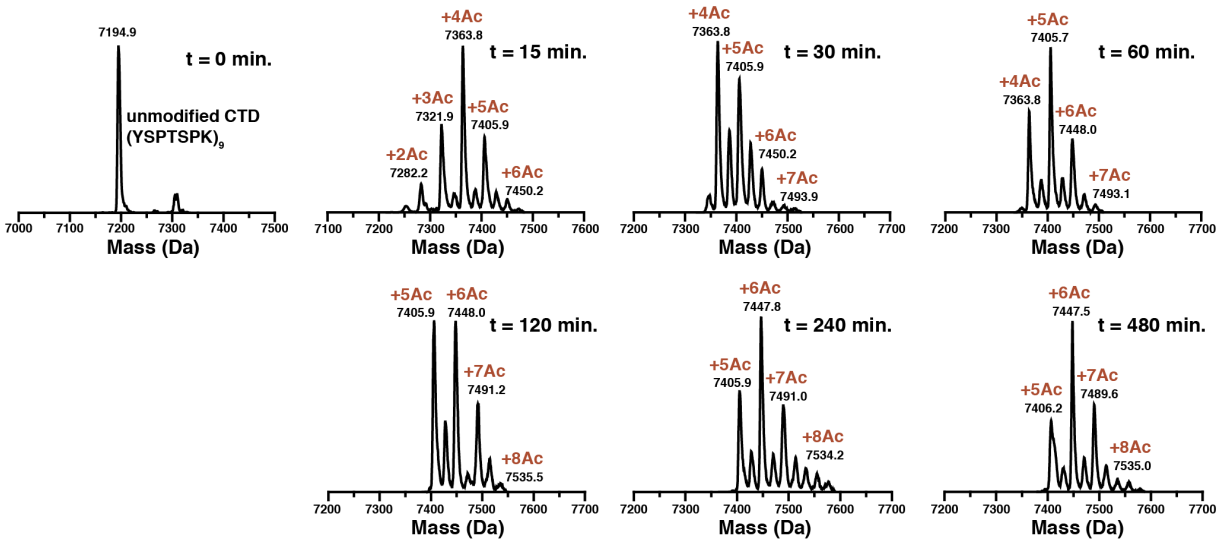

Figure S2: Time course of p300-mediated acetylation of K7 repeats *in vitro*, related to Figure 2

A synthetic CTD peptide of nine repeats, each containing lysines at position 7 $(YSPTSPK)_9$, was acetylated by p300 in a time course experiment and incorporated up to eight acetyl groups as determined by ESI mass spectrometry. Time points of the reaction were taken after 15, 30, 60, 120, 240, and 480 min.

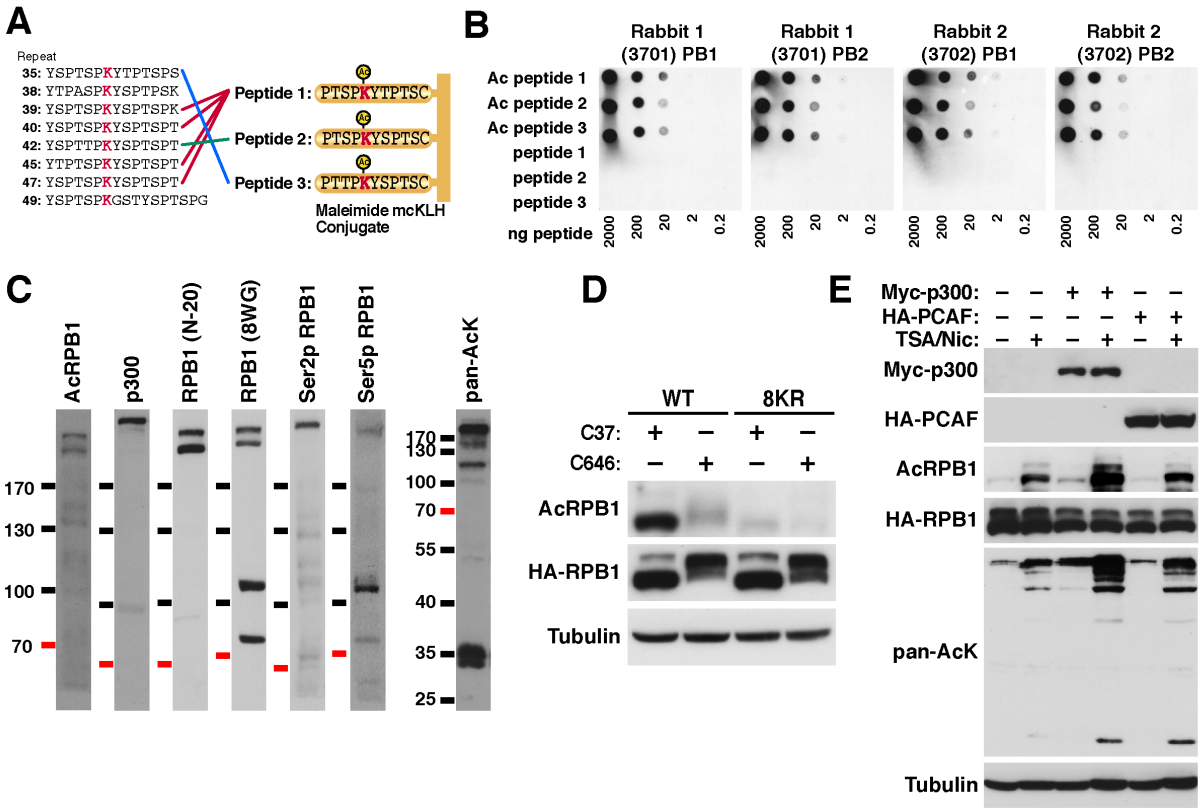

Figure S3: Generation of polyclonal antibodies recognizing acetylated RPB1, related to Figure 3

(A) Peptides used for the generation of the AcRPB1 antibody. **(B)** Dot blots of acetylated and non-acetylated peptides with different production bleeds (PB) of two rabbits immunized with all three acetylated peptides. The non-purified AcRPB1 antibodies recognize all three acetylated peptides but do not cross-react with the non-acetylated peptides. **(C)** Western blot of total cell lysates with different RPB1, p300 (all on 7.5% gels) and pan-AcK antibodies (10% gel) with the molecular weight markers shown for each blot. AcRPB1 antibodies do not cross-react with auto-acetylated p300 and recognize both forms of RPB1. Weak additional bands are observed in the AcRPB1, 8WG, Ser2p and Ser5p blots representing possible degradation products of RPB1. **(D)** Representative western blot of wildtype or 8KR HA-RPB1 expressed in 293T cells cultured in α-amanitin and treated with control substance (C37) or a p300/Kat3B-specific inhibitor (C646) at a concentration of 30 µM for 2 hours. Treatment with C646 decreases acetylation of wild-type HA-RPB1 as expected and in addition causes hyperphosphorylation of wild-type and 8KR HA-RPB1. The mechanism of this hyperphosphorylation is not known but is obviously unrelated to K_7 acetylation. **(E)** Representative western blot of 293T cells expressing wildtype HA-RPB1 transfected either with Myc-tagged p300 or HA-tagged PCAF for 48 hours and then treated with the HDAC inhibitors trichostatin A (TSA) and nicotinamide (Nic) or vehicle controls for 2 hours. Inhibition of deacetylation causes hyperacetylation of HA-Pol II, which is further enhanced by overexpression of p300 (lanes 2 and 4). HA-Pol II acetylation does not increase when PCAF is over-expressed (lane 6). Over-expression of both enzymes induces hyperacetylation of additional cellular proteins as detected by western blotting with a pan-AcK antibody.

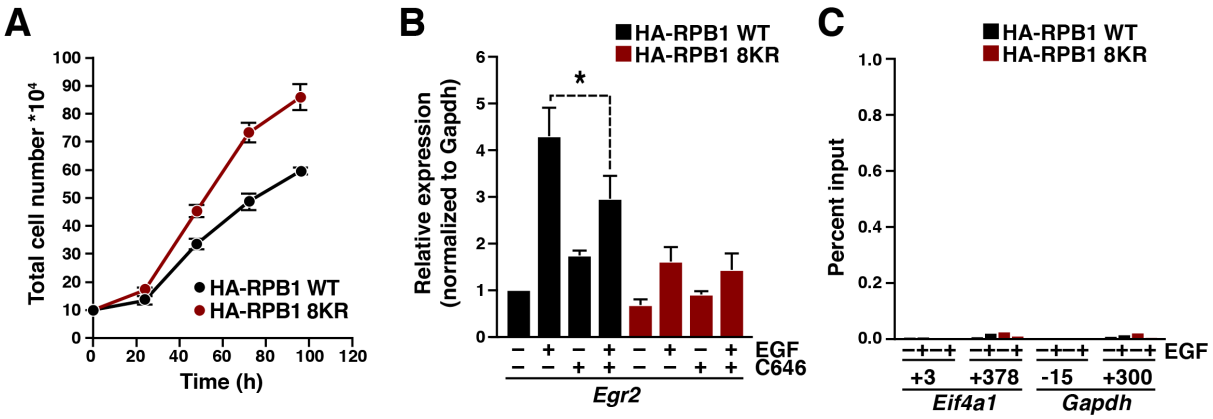

Figure S4: Gene expression in NIH/3T3 cells expressing WT and 8KR mutant HA-RPB1, related to Figure 6

(**A)** Growth curve of NIH/3T3 fibroblasts stably expressing WT or 8KR HA-RPB1 (mean ± s.e.m.; n = 3). **(B)** Real-time RT-PCR analysis of *Egr2* gene expression in NIH/3T3 cells stably expressing WT or 8KR HA-RPB1 treated with the p300/CBP-specific inhibitor C646 for 1 hour followed by stimulation with EGF for 30 minutes. Results are shown as fold expression over untreated WT HA-RPB1 normalized to *Gapdh* (mean ± s.d.; n = 4; *p < 0.05). **(C)** ChIP analysis of *Eif4a1* and *Gapdh* genes performed with no antibodies to confirm specificity of the assay. Primer locations relative to the transcription start site are cited. Data are expressed relative to PCR values obtained in input chromatin solutions before ChIP and represent the average of two ChIPs and duplicate PCRs.

Supplemental Experimental Procedures

Plasmids

The mutant 8KR RNA polymerase II construct was generated by replacing all lysine residues within the RNA polymerase II CTD with arginines, the CTD from the wildtype construct was subcloned into a pBluescript II SK+ vector (Stratagene), with ClaI and EagI restriction sites. Mutagenesis was achieved using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene), according to the supplier's instructions. Primer sequences are available upon request. The mutated CTD was re-introduced into the full-length, parental HA–tagged RPB1 construct.

Immunoprecipitation and Western Blotting

Proteins were immunoprecipitated from cell lysates with 40 µl of equilibrated 50% agarose slurry and the appropriate antibody for 2–10 hours at 4 °C. The beads were washed three times in p300 lysis buffer and resuspended in an equal volume of 2x Laemmli buffer (final concentration: 62.5 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 10 µl/ml 2-mercaptoethanol and 0.6 mg/ml bromophenol blue). Immunoprecipitated cell lysates or *in vitro* modified proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare). After incubation in primary and secondary antibodies, proteins were visualized by chemiluminescent detection using ECL and AmershamHyperfilm ECL (GE Healthcare).

Expression and Purification of Recombinant Proteins

GST-fusion proteins were expressed in *Escherichia coli* BL21(DE3)pLysS (Invitrogen). Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (Sigma) to a final concentration of 100 µM. The bacteria were lysed in PBS containing 1% Triton X-100 (Sigma) and 1 mM PMSF (Calbiochem) by sonification, and GST-fusion proteins were purified using glutathione Sepharose 4B (GE Healthcare).

The extraction from bacteria and purification of the GST-p300-KAT construct used in the *in vitro* acetylation reactions with the GST-CTD was carried out as described (Dormeyer et al., 2003).

GST-CTD proteins were purified immediately prior to being used in *in vitro* acetylation reactions. Beads were equilibrated in washing buffer A (120 mM NaCl, 0.5% NP-40, 50 mM Tris/HCl, pH 8.0) and incubated with the bacterial lysate for 2 hours, washed twice with washing buffer A containing 0.075% SDS, twice with washing buffer A containing 1 mM DTT, twice with washing buffer A and twice with acetylation buffer (50 mM HEPES, pH 8.0, 10% glycerol, 1 mM DTT and 10 mM Na butyrate).

GST-p300 and CTD-K_7 ($(YSPTSPK)_9$) proteins were expressed in *E. coli* and purified by GST affinity purification and size-exclusion chromatography as described elsewhere, with or without TEV protease cleavage and removal of the GST tag (Ott et al., 1999; Schulte et al., 2005).

Recombinant His-Cdk9 was expressed in baculovirus-infected *Sf21* cells for 3½ days at 27 °C after infection with 1:50 of baculovirus carrying the pFastBack-HTb bacmid. Cdk9-expressing cells were resolved for his-tagged proteins in 50 mM HEPES, pH 7.6, 500 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and protease inhibitor cocktail, and lysed by sonification. To reconstitute the P-TEFb complex, recombinantly expressed and purified CycT1 (1-272, Schulte *et al.,* 2005) was added before and after cell disruption. After centrifugation for 2x40 min at 30,000xg, the complex was isolated from the supernatant using Ni-NTA affinity chromatography (GE Healthcare) in washing buffer (50 mM HEPES, pH 7.6, 500 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, using a linear gradient from 20 to 400 mM imidazole). Protein-containing fractions were

concentrated and further purified in washing buffer by gel filtration using a S200 16/60 size-exclusion column (GE Healthcare).

Supplemental References

Dormeyer, W., Dorr, A., Ott, M., and Schnölzer, M. (2003). Acetylation of the HIV-1 Tat protein: an in vitro study. Anal. Bioanal. Chem. *376*, 994-1005.

Ott, M., Schnölzer, M., Garnica, J., Fischle, W., Emiliani, S., Rackwitz, H.-R., and Verdin, E. (1999). Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. Curr. Biol. *9*, 1489-1493.

Schulte, A., Czudnochowski, N., Barboric, M., Schönichen, A., Blazek, D., Peterlin, B.M., and Geyer, M. (2005). Identification of a Cyclin T-Binding Domain in Hexim1 and Biochemical Analysis of Its Binding Competition with HIV-1 Tat. J. Biol. Chem. *280*, 24968-24977.