Supplementary information

Human U1 snRNA forms a novel chromatin-associated snRNP with TAF15

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Methods

Plasmid constructions

TAF15 cDNA from pTL1 using BamHI and XhoI sites has been cloned into the pSG5-puro-flagNt vector (Green et al., 1988). Point mutants were generated by PCR using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). All constructs were verified by DNA sequencing.

Cell treatments

HeLa cells were incubated with 20 μg/ml α-amanitin (Roche) for 3 hours.

Nuclear extract preparation

Nuclear extracts were prepared as previously described in (Bertolotti et al., 1999).

Immunoprecipitation, Western blot analysis and antibodies

Proteins from 500 μg of nuclear extract were immunoprecipitated with 50 μl protein G-Sepharose (Pharmacia) and approximately 5-10 μg of the different antibodies as described (Bertolotti et al., 1998), except that IP buffers containing NaCl instead of KCl were used. Chemiluminescence detection was performed according to manufacturer's instructions (Amersham). Monoclonal antibodies (mAb) directed against TAF15 (7TA2B11 and 8TA2B10) and against TAF5 (2D2) and TBP (3G3) have been described (Bertolotti et al., 1996; Bertolotti et al. 1998). Anti-U1-70K (Billings et al., 1982), anti-SmD recognizing SmD1 and D3 proteins (Billings et al., 1985), anti-Sm B/B' D (Y12 mAb, Abcam) and anti-GAL4 (2GV3) (White et al., 1992) monoclonal antibodies have been described previously. Anti-U1-A (Dumortier et al., 1999) and anti-U1-C (Dumortier et al., 1998) rabbit polyclonal antibodies were a kind gift from Dr. S. Muller. The anti-histone H3 (1HH3-3E1 clone) monoclonal antibody (Euromedex) was raised against the residues 8 to 19 of the human histone H3 protein. The anti-cap antibodies, TMG and H-20, were kindly provided by Dr. R. Lührmann.

RNA-immunoprecipitation (RNA-IP) assay

RNA-IP assay was performed as described (Martianov et al., 2007). About $4x10^7$ HeLa cells were crosslinked with 1% formaldehyde for 10 min. The crosslinking reaction was stopped by adding glycine to 330 mM final concentration. Cells were re-suspended in 500 µl RIP lysis buffer (50 mM HEPES, pH7.5, 1 mM EDTA, 1% triton) and sonicated 10 times for 10 sec. Sample was diluted and adjusted to 0.5% triton, 25 mM $MgCl_2$, 5 mM $CaCl_2$ and DNA was digested with RNase free DNase (30 units) at 37°C for 15 min. Digestion was stopped with 20 mM EDTA. Sample was pre-cleared with protein G-Sepharose beads for 2 hours at 4°C and followed by addition of 10 µg of antibodies. Twenty µl of protein G-Sepharose beads were added after 2 hours of incubation and left for another 1 hour at 4°C. Beads were washed once with 1 ml binding buffer (50 mM HEPES, 0.5 % triton, 25 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM EDTA), with FA500 (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1% triton, 0.1 % Na deoxycholate), with LiCl buffer (10 mM Tris, 250 mM LiCl, 1 % triton, 0.5 % Na deoxycholate, 1 mM EDTA) and finally, with TES (10 mM Tris, 10 mM NaCl, 1 mM EDTA). Immunoprecipitates were eluted with 75 µl RIP elution buffer (100 mM Tris pH 7.8, 10 mM EDTA, 1 % SDS). NaCl was adjusted to 200 mM and the samples were treated with 20µg of proteinase K for 1 hour at 42°C and 1 hour at 65°C. RNA was extracted with RNA Solv® reagent and analyzed by RT-PCR.

Reverse transcription (RT) and real time quantitative PCR (qPCR)

Total RNA was isolated with RNA Solv® reagent (Omega Bio-Tek), precipitated and used as a template for reverse transcription with random hexamer primers and M-MLV reverse transcriptase (Sigma) following the manufacturer's instructions. $1/25^{th}$ of each RT product was amplified with the QuantiTect SYBR Green PCR kit (Qiagen). Quantitative PCR were performed on a Light Cycler apparatus (Roche).

RNA extraction and analysis of TAF15-bound nucleic acids

Preparation of HeLa cell nuclei was performed according to (Tyc and Steitz, 1989). RNAs from HeLa cells and from the nuclear fraction of HeLa cells were isolated by the guanidinium thiocyanate/phenol-chloroform extraction method (Goodall et al., 1990). RNA 3' end labelling with [5'-^{32}P]pCp and T4 RNA ligase (BioLabs) and RNase A/T1 protection assay were preformed as described (England et al., 1980, Goodall et al., 1990). To generate sequence-specific antisense RNA probes, recombinant pBluescribe plasmids carrying full lenght cDNAs of the human U1, U2 or U4 snRNAs were linearized with the appropriate restriction endonucleases and used as templates for *in vitro* transcription with the T7 RNA polymerase (Promega) in the presence of [alpha-^{32}P]CTP (specific activity, 30 Ci/mmol). Before utilization, all probes were purified on a 6% sequencing gel.

Primer extension mapping

Primer extension mappings of ribose-methylated nucleotides and pseudouridines have been described (Kiss and Jády, 2004).

Preparation of nuclei and salt extraction

HeLa cells were harvested, washed twice with ice-cold PBS and resuspended in 15 mM Tris-HCl buffer pH 7.5, containing 60 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 250 mM sucrose, protease inhibitor cocktail (PIC) and 0.5 mM PMSF (Buffer N). An equal volume of Buffer N containing 0.6% NP-40 was added to the cells and the suspension was gently mixed and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 2,000 g for 5 min at 4°C and washed twice with Buffer N before final suspension in the same buffer. DNA was estimated by measuring UV absorption at 260 nm (A_{260}) in a 2 M NaCl solution.

HeLa nuclei were incubated in 100 µl Buffer N containing 75, 200, 350, 500 and 1000 mM NaCl in the absence of sucrose. After incubation for 30 min on ice, the samples were centrifuged at 12,800 g for 15 min at 4°C to yield the soluble and insoluble (pellet) fractions.

Equivalent samples from each fraction were subjected to SDS-PAGE and Western blot analysis. Salt extraction in the presence of RNase A was done by adding 1 μg/μl RNase A in Buffer N.

Chromatin isolation

One gram of pelleted HeLa cells were lysed in 1 ml hypotonic buffer (10 mM Tris-HCl, pH 7.7, 1.5 mM $MgCl_2$, 10 mM KCl) and subjected to dounce homogenization (8 strokes) using pestle B to release nuclei. Then, 0.33 ml of Sucrose Buffer (20 mM Tris-HCl, pH 7.7, 15 mM KCl, 60 mM NaCl, 0.34 M sucrose, 0.15 mM spermine, 0.5 mM spermidine) was added. Cytoplasm was separated from nuclei by centrifugation at 9,000 g for 10 min at 4°C. The nuclei pellet was resuspended in 1 ml of sucrose buffer. Nucleoplasm was separated from chromatin by extraction with 0.35 ml of high salt buffer (20 mM Tris-HCl, pH 7.7, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 900 mM NaCl) for 30 min at 4°C and centrifugation at 9,000 g for 10 min. The chromatin pellet was resuspended in 1 ml of sucrose buffer and digested with 2.5 units of micrococcal nuclease (Sigma, N3755) in the presence of 1 mM $CaCl_2$. Digestion was stopped by the addition of 4 mM EDTA. The digested extract was sonicated to shear chromatin (5 pulses, 20 seconds on, 20 seconds off on a VibraCell 72412 sonicator, Bioblock Scientific). The obtained cytoplasmic, nucleoplasmic and chromatin fractions were centrifuged at 12,000 rpm and dialyzed overnight against IP buffer containing 150 mM NaCl.

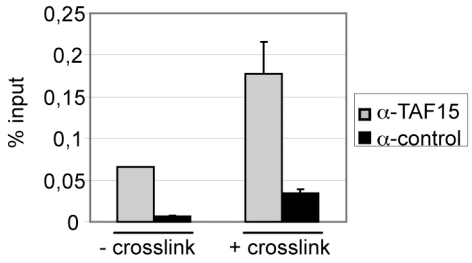
Gel filtration chromatography

A HeLa chromatin extract (10 μg/μl) was loaded on a Superose 6 size exclusion chromatography column using the SMART FPLC system (Pharmacia) and separated at a flow rate of 40 μl/min in buffer A (50 mM Tris-HCl, pH 7.9, 0.1 M NaCl, 1 mM EDTA, 0.005% NP-40, 1 mM DTT). Forty-eight fractions of 50 μl were collected and even fractions analyzed by Western blot.

Immunofluorescence and *in situ* hybridization

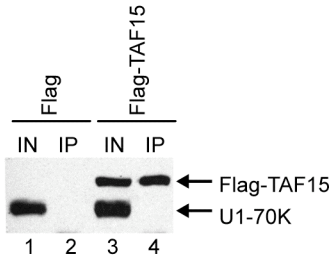
Fixation, permeabilization, immunostaining and *in situ* hybridization of HeLa cells as well as image acquisition and processing were performed as described (Darzacq et al., 2002). Synthesis and chemical conjugation of amino-modified oligonucleotides with Fluoro-Link Cy3 and Cy5 monofunctional dyes (Amersham) were performed according to the protocols of the laboratory of R.H. Singer (http://singerlab.aecom.yu.edu). The following oligonucleotide probes were used to detect U1 (C*CGGAGTGCAATGGAT*AAGCCTCGCCTGGG*) and U2 (AT*ACTGATAAGAACAGATACT*ACACTTGATCTTAGCCAT*A) snRNAs. Amino-allyl-modified residues are indicated by asterisks. Human TAF15, U1-70K and Sm proteins were detected by monoclonal anti-TAF15 (7TA2B11), anti-U1-70K and anti-Sm (Y12) antibodies, followed by incubation with anti-mouse antibodies conjugated to fluorescein (Sigma). HeLa nucleoli were visualized by transient expression of a recombinant fibrillarin-GFP protein (Dundr et al., 2000). Nuclear DNA was stained with 0.1 μg/ml DAPI.

Supplementary Figures



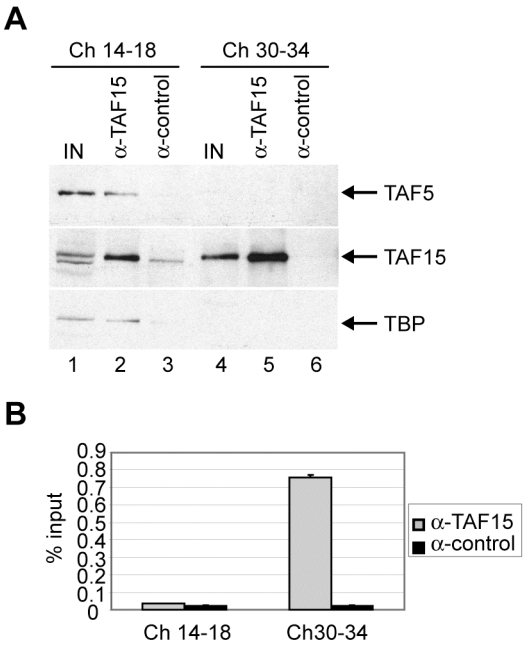
Supplementary Figure 1

In vivo RNA-IP assay. HeLa cells were either treated (+) or not treated (-) with 1% formaldehyde to form *in vivo* RNA-protein crosslinks. After extract preparation and TAF15 IP, the co-immunoprecipitated RNA was purified and analysed by reverse transcription followed by real time quantitative PCR (RT-qPCR) with U1 snRNA-specific primers. Quantitative PCRs were done in duplicates.



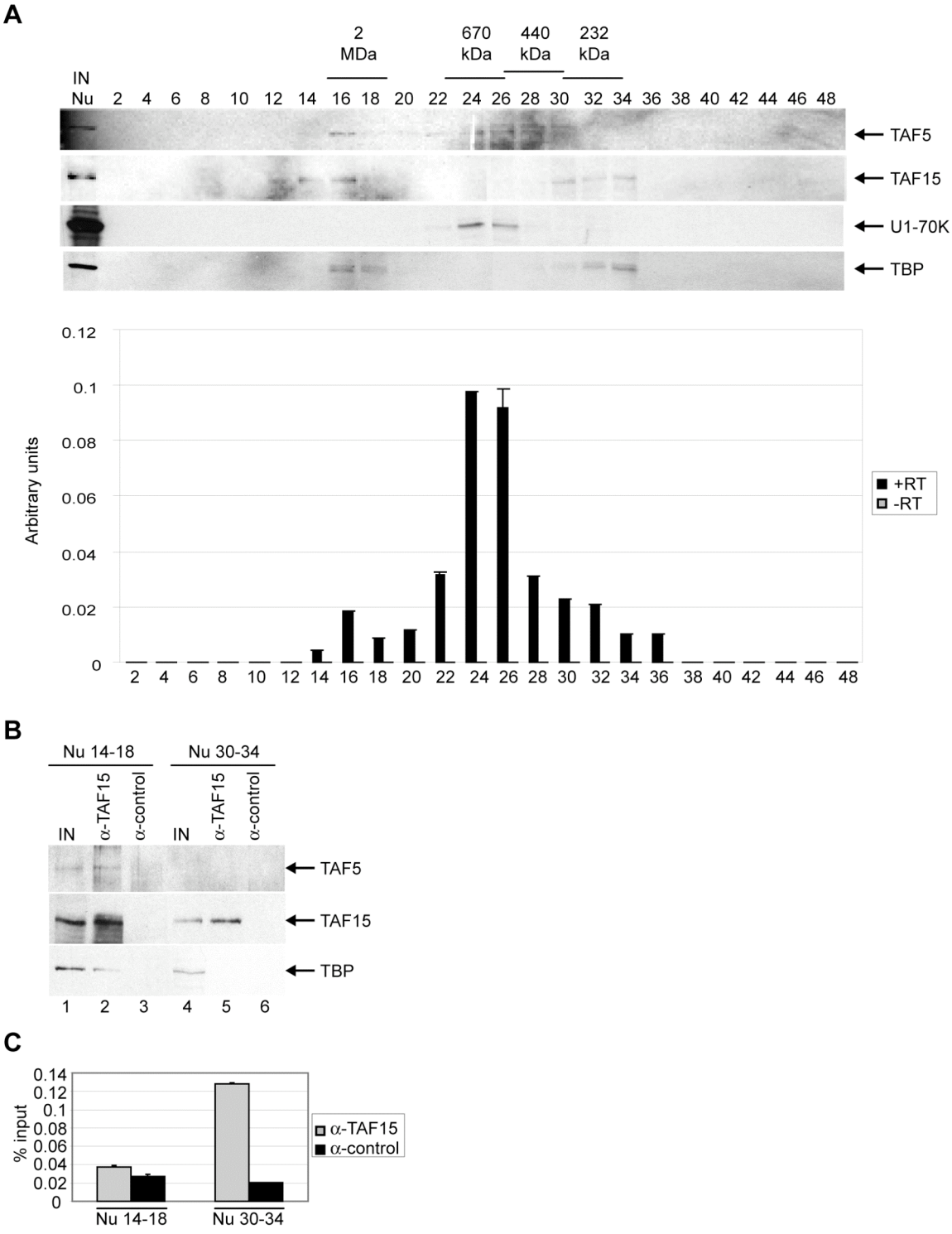
Supplementary Figure 2

HeLa cells were transfected with an empty vector (Flag) or with a vector expressing the Flag-TAF15 recombinant protein. Forty-eight hours after transfection, nuclear extracts (IN) were prepared and subjected to immunoprecipitation (IP) using an anti-Flag antibody. The Flag-TAF15 and U1-70K proteins were detected by Western blot analysis.



Supplementary Figure 3

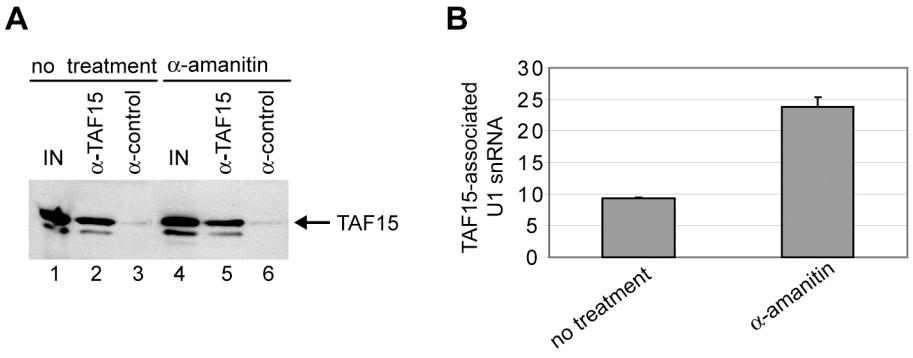
Detection and characterization of two distinct TAF15-containg complexes in HeLa chromatin-soluble extract. (**A**) Fractions 14 to 18 or 30 to 34 obtained upon size fractionation of HeLa chromatin-soluble extract (see Figure 4E) were pooled and subjected to IP with either anti-TAF15 or control antibodies. Co-purification of TFIID subunits TAF5 and TBP was analyzed by Western blot. TAF15 associates with TFIID in the high molecular size fractions (14-18), but not in small molecular weight fractions 30-34. (**C**) Fractions 14 to 18 or fractions 30 to 34 (Figure 4E) were pooled and subjected to IPs with either anti-TAF15 or control antibodies. Co-precipitation of U1 snRNA was measured by RT-qPCR. TAF15 associates with U1 snRNA only in the low molecular weight fractions 30-34.



Supplementary Figure 4

Size fractionation of a HeLa nucleoplasmic extract reveals two distinct TAF15-containing complexes. (**A**) HeLa nuclear soluble extract was fractionated by gel filtration. The input (IN Nu) and even fractions were analysed by Western blot with the indicated antibodies. The

elution profile of molecular mass markers is indicated. The presence of U1 snRNA in each fraction was measured by RT-qPCR with or without reverse transcriptase (RT) (lower panel). The error bars represent the variations between triplicate measurements. (**B**) Fractions 14 to 18 or 30 to 34 (from panel A) were pooled and subjected to IPs with either anti-TAF15 or control antibodies. Co-purification of TFIID subunits TAF5 and TBP was analyzed by Western blot. TAF15 associates with TFIID in fractions 14-18, but not in 30-34. (**C**) Fractions 14 to 18 or 30 to 34 (from panel A) were pooled and subjected to IP with either anti-TAF15 or control antibodies. Co-precipitation of U1 snRNA was measured by RT-qPCR. TAF15 associates with U1 snRNA in fractions 30-34, but not in 14-18.



Supplementary Figure 5

(**A**) From HeLa cells, either not treated or treated with 20 μg/ml α-amanitin for 3 hours, nuclear extracts (IN) were prepared and subjected to IP performed with TAF15-specific and control antibodies. The immunoprecipitated TAF15 was analysed by Western blot. (**B**) The TAF15-associated U1 snRNA was monitored by RT-qPCR. The measured values are represented as fold enrichments when compared to the background values obtained with the control antibodies.

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