Proteomic Analysis of Mitotic RNA Polymerase II Reveals Novel Interactors and Association With Proteins Dysfunctional in Disease*

André Möller†, Sheila Q. Xie‡, Fabian Hosp§, Benjamin Lang¶, Hemali P. Phatnani §§, Sonya James¶¶, Francisco Ramirez**, Gayle B. Collin‡‡, Jürgen K. Naggert‡‡, M. Madan Babu¶¶, Arno L. Greenleaf¶¶, Matthias Selbach§, and Ana Pombo‡

RNA polymerase II (RNAPII) transcribes protein-coding genes in eukaryotes and interacts with factors involved in chromatin remodeling, transcriptional activation, elongation, and RNA processing. Here, we present the isolation of native RNAPII complexes using mild extraction conditions and immunoaffinity purification. RNAPII complexes were extracted from mitotic cells, where they exist dissociated from chromatin. The proteomic content of native complexes in total and size-fractionated extracts was determined using highly sensitive LC-MS/MS. Protein associations with RNAPII were validated by high-resolution immunolocalization experiments in both mitotic cells and in interphase nuclei. Functional assays of transcriptional activity were performed after siRNA-mediated knockdown. We identify >400 RNAPII-associated proteins in mitosis, among these previously uncharacterized proteins for which we show roles in transcriptional elongation. We also identify, as novel functional RNAPII interactors, two proteins involved in human disease, ALMS1 and TFG, emphasizing the importance of gene regulation for normal development and physiology. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.011767, 1–16, 2012.

The ability of cells to transcribe specific subsets of mRNAs relies on activities influencing the accessibility of gene promoters within their chromatin context, the formation of pre-initiation complexes, the initiation of RNA synthesis, and the transition to states of productive elongation (1). Central to these activities is the RNAPII complex itself, which interacts with chromatin regulators, transcription initiation factors, and with complexes required for processive elongation (2). Furthermore, chromatin-bound RNAPII recruits enzymatic activities involved in the cotranscriptional processing of nascent RNA, such as capping, splicing, and 3' processing of primary transcripts (3).

Recruitment depends on specific phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNAPII, RPB1, consisting of a highly repetitive heptapeptide sequence (Y1S2P3T4S5P6S7; 52 repeats in mammals). S2, S5, and S7 residues become phosphorylated during transcription: S5 and S7 during the stage of transcriptional initiation, and S2 during transcriptional elongation (4).

The regulation of transcription and its downstream processes is of central importance in many human diseases (5, 6), and many proteins involved in these processes are aberrantly expressed or dysfunctional in cancer (7, 8). Comprehensive knowledge of the mammalian RNAPII protein interaction landscape may therefore further our understanding of disease genesis in humans, and ultimately help prevent its progression.

In this study, we have purified intrinsically soluble RNAPII from mitotic cells, a strategy based on observations that RNAPII dissociates from chromatin as the cell enters mitosis ((9); Fig. 1A), and that functionally relevant components, such as splicing factors, can remain associated with RNAPII during mitosis (10). We speculated that other cotranscriptional interactions with RNAPII may also be transcription-independent and more stable than anticipated, and that identification of the RNAPII proteome in native conditions during mitosis would clear cells; PFA, paraformaldehyde; PHA, phytohaemagglutinin; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; RNAPIIα, hypophosphorylated RNAPII; RNAPIIβ, hyperphosphorylated RNAPII; SD, standard deviation.

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allow the identification of novel RNAPII-associated proteins. We combined low stringency lysis and immunoprecipitation using highly specific elution conditions with mass spectrometry and appropriate control purifications. We show that mitotic RNAPII is present in complexes with sizes expected for the canonical 12 subunit complex (~700 KDa) and in much larger (≧2 MDa) complexes, enriched in S2 and S5 phosphorylation. Our mass-spectrometric analyses of complexes from total and size-fractionated lysates revealed >400 RNAPII associated proteins, including novel and known RNAPII interactors. We have validated various interactions by in situ experiments using fluorescence microscopy, measuring transcriptional activity after RNAi and colocalization between RNAPII and candidate interactors. The results allow us to infer a functional importance for several of the RNAPII interactors in interphase, including proteins that have been implicated in human disease.

**EXPERIMENTAL PROCEDURES**

**Mitotic Cell Collection, Lysis, Gel Filtration and Immunoprecipitation**—Mitotic cells were collected and frozen in conditions that preserve viability. Mitotic cell lysates cleared of chromosomes and larger organelles were treated with calf intestinal phosphatase and Turbo DNase, unless otherwise stated. Fractionation of mitotic lysates was performed on a Superose 6 10/300 GL column (GE Healthcare).

For immunoprecipitation of RNAPII associated proteins, total lysate, fraction 2 or fraction 8 samples were incubated with antibody 8WG16 (mouse ascites, Covance, Princeton, NJ) or control mouse IgG2a antibody (monoclonal cell culture supernatant; DakoCytomation). Proteins were eluted by resuspension in elution buffer containing a CTD repeat peptide (ab12795; Abcam, Cambridge, MA) and ethanol precipitated. Full details are available in Supplementary Text.

**Mass Spectrometry**—Immunoprecipitated protein pellets were solubilized in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0) buffer, reduced with 1 mM dithiothreitol, alkylated with 5.5 mM iodoacetamide and digested using modified lysyl endopeptidase (LysC; 100 w/w, Wako, Osaka, Japan) and subsequently sequencing grade modified trypsin (1:100 w/w, Promega, Mannheim, Germany) in 50 mM ammonium carbonate buffer. Peptides were purified using stop and go extraction (STAGE) tips (11). Liquid chromatography-tandem MS (LC-MS/MS) analysis was performed online as previously described (12). In brief, peptide mixtures were separated by reversed phase chromatography using the Eksigent NanoLC - 1D Plus system (Eksigent, Dublin, CA) on in-house manufactured 10 cm fritless silica microcolumns with an inner diameter of 75 μm. Columns were packed with ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Ammerbuch, Germany) (13). Separation was performed using a 10–60% acetonitrile gradient (155 min) with 0.5% acetic acid at a flow rate of 500 nL/min. Eluting peptides were directly ionized by electrospray ionization and transferred into the orifice of a LTQ-Orbitrap classic hybrid mass spectrometer (Thermo Fisher, Waltham, MA). Mass spectrometry was performed in the data dependent mode with one full scan in the Orbitrap (m/z = 300–1700; r = 60,000; target value = 1 × 105). The five most intense ions with a charge state greater than one were selected (target value 5000; monoisotopic precursor selection enabled) and fragmented in the LTQ using collision-induced dissociation (35% normalized collision energy, wide-band activation enabled). Dynamic exclusion for selected precursor ions was set to 60 s. The MaxQuant software package (version 1.0.12.5) was used to identify proteins (14, 15) with enabled polymer detection and top six MS/MS peaks selection per 100 Da. Peak lists were searched on a MASCOT search engine (version 2.2.3, MatrixScience, Boston, MA) against an in-house curated concatenated target-decoy database (16) of forward and reversed proteins in the International Protein Index (IPI) human protein database (version 3.43, 72,346 entries), supplemented with common contaminants. Carbamidomethylation of cysteine was selected as fixed modification, oxidation of methionine and acetylation of the protein N terminus were used as variable modifications. Trypsin/p + DP was selected as protease (full specifically) with a maximum of three missed cleavages. Mass tolerance for fragment ions was set to 0.5 Da. The original mass tolerance of precursor ions was 7 p.p.m., but narrowed down further to the individual mass tolerances calculated by MaxQuant. A minimum of six amino acids per identified peptide and at least one peptide per protein group were required. Only unique peptides or peptides assigned to the protein group with the highest number of peptides are reported. False discovery rate was set to 1% at both the peptide and protein level. The data associated with this study may be downloaded from Proteome Commons (http://proteomecommons.org) Tranche using the following hash: MfbhAHHSVh3icV8sHJp-IYNFUh1uw9csegsyofm0BHm08vnMFApMzsztKQjxgjhnUJ6Kcb7SFz-LA6HrFk9r5H475s5oAAAHsHg==

Gene Ontology (GO) Analysis—Over- and under-representation of GO terms was analyzed using GOstat (17) in conjunction with the goa_human annotation data set and Benjamini-Hochberg correction for multiple testing.

RNAi and Br-UTP Incorporation Assay—HeLa cells were cotransfected with two different siRNAs against each gene of interest or with control siRNA using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). At 72 h post-transfection, in situ transcription run-ons were performed in the presence of Br-UTP and labeled Br-RNA indirectly immunolabeled as described in (18). Confocal microscopy images were analyzed using ImageJ software. SirNA results were analyzed by ANOVA (using SAS, version 9.1), testing for all main effects (siRNA treatment, day of transfection and Br-UTP labeling set) of which only the siRNA treatment and day of transfection were significant (both p < 0.0001). The ANOVA estimates were used to compare siRNA effects relative to control siRNA using Student’s t test. The p values represented in Fig. 4 are adjusted for multiple testing using the method of false discovery rate by Benjamini and Hochberg. Full details are available in supplementary Text.

**Protein Immunolocalization And Antibody Blocking Assay (ABA)**

Using Tokuyasu Cryosections—HeLa cells, human PHA-activated peripheral blood mononuclear cells (PBMCs), and pancreatic and hypothalamic mouse tissues were used for ABA. Human female PBMCs were purified using the Leuco-Sep kit (human; Harlan Sera-Lab, Loughborough, United Kingdom) and activated with phytohemagglutinin (PHA; Sigma) for 72 h. PBMCs and cultured HeLa cells were fixed in 4% paraformaldehyde (PFA; EM-grade) in 250 mM HEPES (pH 7.6; 10 min and 2 h, respectively). Mice used in the study were maintained in the Research Facility at The Jackson Laboratory. Experimental procedures were carried out under Institutional Animal Care and Use Committee approval. Briefly, pancreatic and hypothalamic tissues were collected following intracardiac perfusion with 4% PFA/HEPES solution. Tissues were dissected into 1.5 mm pieces in cold 4% PFA/HEPES solution and fixed for an additional 2 h 8% PFA/HEPES. Fixed cell pellets and dissected tissues were embedded in sucrose solution and frozen in liquid nitrogen. Cryosections (150 nm thick) on coverslips were processed for immunofluorescence labeling or ABA, as previously described (18, 20).
For ABA, cryosections were pretreated with alkaline-phosphatase to ensure maximized binding of 8WG16 to detected total RPB1, and processed as previously described (20).

Confocal microscopy images from cryosections were collected using settings calibrated on the negative control (no antibody) samples without saturation of the intensity signal. Nuclear fluorescence intensities were determined in Photoshop. Statistical significance was tested by ANOVA (using SAS, version 9.1). ANOVA estimates were used to compare the blocking effects of antibodies to proteins of interest relative to control blocking antibodies using Student’s t test. The p values represented in Figs. 3 and 4 are adjusted for multiple testing using the method of false discovery rate by Benjamini and Hochberg. Full experimental details and primary antibodies used are available in Supplementary Text.

RESULTS

**RNAPII is Present in ≈2 MDa Complexes During Mitosis**—To enhance our understanding of the protein interaction landscape of RNAPII, we purified RNAPII complexes under mild conditions from mitotic cells, where RNAPII is mostly excluded from the condensed chromosomes and distributed throughout the mitotic cytoplasm (Fig. 1A).

Mitotic cells were lysed in the presence of the natural, nonionic surfactant saponin, which preserves RNAPII function during interphase (18), and using a near physiological salt concentration of 150 mM NaCl. The resulting lysate was treated with DNase to remove residual DNA, which might otherwise act as a scaffold for indirect protein-protein interactions (Fig. 1B). The lack of chromatin contamination was confirmed by testing for the presence of histone H2B in the mitotic lysate (Fig. 1C). Although H2B is detectable in whole cell lysates of unsynchronized cells (95% interphase), no H2B is detectable in the mitotic lysate clarified of chromosomes.

To investigate the size of RNAPII complexes in mitosis, we performed size fractionation of the mitotic lysate followed by Western blotting using various antibodies directed against subunits of RNAPII (RPB1, RPB2, and RPB3) and CTD phospho-epitopes (S2p and S5p; Fig. 1D). We identify RNAPII enrichment in two parts of the elution spectrum: at ≈700 KDa (around fraction 8), a size expected for the canonical 12 subunit RNAPII complex, and at ≈2 MDa (fraction 2). RNAPII complexes in the 2–4 MDa range have previously been reported in whole cell and nuclear extracts from asynchronous populations of mammalian cells (21–23), but not from mitotic cells, and were shown to contain general transcription factors (GTFs) and mediator subunits as well as DNA repair proteins (21) or transcription elongation factors (22).

Interestingly, using antibodies directed against phosphorylated RPB1, we detect an enrichment of hyperphosphorylated RPB1 in the larger RNAPII containing complexes, suggesting that RPB1 phosphorylation in mitotic cells may be associated with the formation of higher-order complexes between RNAPII and additional factors. This mirrors current models for the role of RNAPII phosphorylation during the transcription cycle in recruiting specific chromatin remodelers and the RNA processing machinery (3, 4).

Scheme for the Purification of RNAPII Complexes From Mitotic Extracts—To purify RNAPII-containing protein complexes from total and size-fractionated extracts, we devised an immunoprecipitation strategy (Fig. 1E) based on the binding of the antibody 8WG16 to the CTD of RPB1 (24). This is followed by elution using a CTD peptide consisting of two repeats of the CTD heptad sequence that effectively competes for binding to the 8WG16 paratope and specifically elutes RNAPII complexes from 8WG16-bound agarose beads post-immunoprecipitation (Fig. 1F). The highly repetitive nature of the CTD allows for a high-affinity interaction with 8WG16. Because of the known propensity of 8WG16 to preferentially bind hypophosphorylated RPB1 (19, 25, 26), we initially introduced an alkaline phosphatase (AP) treatment into our purification strategy (19, supplementary Fig. S1). Subsequently, we investigated RNAPII interactors in the absence of AP treatment (supplementary Fig. S2). As an additional specificity control, we performed parallel immunoprecipitations with a matched isotype control antibody. Six different samples were initially analyzed by mass-spectrometry from AP-treated mitotic extracts: the pre-fractionation (total) lysate, and the gel filtration fractions containing the ≈2 MDa (fraction 2) or the ≈700 KDa (fraction 8) RNAPII complexes, together with their three matched isotype controls.

**Mass-Spectrometric Identification of RNAPII-associated Proteins**—To investigate RNAPII-associated proteins in mitosis, we first purified the total mitotic lysate directly, without size fractionation. The eluates from 8WG16 and control IPs were subsequently trypsinized and analyzed by LC-MS/MS. A total of 511 proteins were identified in the 8WG16 sample, of which 387 proteins were represented by at least two unique peptides (Table I). Half of these proteins (n = 178) were specific to the 8WG16 IP (i.e. not identified in the isotype control IP). A second category of “more abundant” proteins (n = 117), represented with more peptides in the 8WG16 IP than in the isotype control (≥2 peptides difference) were also considered, all of which also exhibit higher mean peptide intensities (2- to 297-fold; average 25-fold; S.D. 41-fold; supplementary Table S1). In total, we identified 295 RNAPII-associated proteins in the mitotic lysate, as either “specific” (i.e. ≥2 peptides in the 8WG16 sample and no identification in the control) or “more abundant” (≥2 peptides difference).

Among the RNAPII-associated proteins in mitosis, we find all twelve subunits of RNAPII and a number of proteins known to directly interact with RNAPII (e.g. GDOWN1 (27), SAFB1 (28), RMP (29), SFPQ, and NONO (30). Previous proteomic studies of RNAPII-associated proteins (31–33) reported only eight (31) or five (32) RNAPII subunits, highlighting the value of milder purification conditions to achieve a more complete characterization of RNAPII complexes.

To further assess the quality of the purification, we compared the abundance of RNAPII-associated proteins relative to RNAPII subunits. As a rough estimate of protein abundance we used the ratio of observed unique peptides to theoretically
observable unique peptides, similar to the protein abundance index (PAI) (34). According to this calculation, the top 21% most abundant proteins lie within the PAI range of the RNAPII subunits (0.6–1.2) and a further 18% have a PAI 0.3–0.6 (at least half the PAI of the least abundant RNAPII subunit; POLR2H, PAI = 0.6). Several proteins of unknown function have PAIs in range of 0.3–1.2 (TFG, MRT4, ZNF326, FAM103A1), whereas the least abundant, known RNAPII interactor identified by our approach has a PAI of 0.08 (SAFB1; (28)), suggesting that several of the RNAPII-associated proteins identified are likely to be bona fide interactors.

To identify proteins specific to larger (>2 MDa) and smaller (<700 kDa) RNAPII complexes, we extended the proteomic analysis to samples from fractions 2 and 8 obtained after gel filtration chromatography.
proteins, including SRCAP, hINO80, and TRRAP/TIP60 (37), RUVB1 and RUVB2 are known constituents of various complexes, including SRCAP, hINO80, and TRRAP/TIP60 (37). Although RUVB1 and RUVB2 are present in the RNAPII subunits (Fig. 2C), they do not appear to interact with RNAPII itself.

We next compared the proteins identified as specific or more abundant in the total lysate and the size fractions. Here, we included specific proteins identified with only one peptide, if their presence in multiple 8WG16 IP samples supported their correct identification. We found 32 proteins to be present in all three samples (Fig. 2B and Table III), including eleven of the twelve canonical RNAPII subunits. Furthermore, 172 proteins were common between the total lysate and fraction 2, and 42 proteins between the total lysate and fraction 8.

### Mitotic RNAPII Complexes are Enriched in Proteins with Known Functions in Transcription, RNA Processing and Translation

To explore the functions of the proteins associated with RNAPII in mitosis, we analyzed the Gene Ontology (GO) terms of the specific and more-abundant proteins identified in the total lysate, fraction 2, and fraction 8 (Fig. 2C). Despite RNAPII being transcriptionally silent and dissociated...
TABLE II
Examples of protein complexes and single proteins identified by mass-spectrometry of native mitotic RNAPII complexes. Values of PAI are normalized to the mean PAI of the RNAPII complex (nPAI). X denotes mean nPAI of identified complex components. The complete list of proteins shared by, or exclusive to, specific fractions and/or the lysate is found in supplementary Table S2.

| Group/Complex | Lysate | Fraction 2 | Fraction 8 |
|---------------|--------|------------|------------|
| **RNAPII**    |        | RPB1, RPB2, RPB3, RPB4, RPB5, RPB6, RPB7, RPB8, RPB9, RPB10, RPB11, RPB12 | RPB1, RPB2, RPB3, RPB4, RPB5, RPB7, RPB8, RPB9, RPB10, RPB11 | RPB1, RPB2, RPB3, RPB4, RPB5, RPB6, RPB7, RPB8, RPB9, RPB10, RPB11 |
| **U1 snRNP**  | SNRP70, SNRPA, SNRPC | X = 0.76 | SF3A1, SF3A3, SF3B1, SF3B2, U2AF1, U2AF2 | X = 0.25 |
| **U2 snRNP**  + associated factors | SNRP1A, SF3B1, SF3B2, SF3B3, SF3A1, SF3A3, SF3B1, SF3B2 | X = 0.19 | - | - |
| **snRNP core** | EFTUD2, PRPB, ASSC3L1 | X = 0.08 | SF3A1, SF3A3, SF3B1, SF3B2, U2AF1, U2AF2 | - |
| **SMN complex** | SNRPDC, SNRP, SNRPF, | X = 0.92 | - | - |
| **URI/Prefoldin** | SMN, GEMIN5, STRAP, DDX20, GEMIN4 | X = 0.20 | - | - |
| **Mediator** | RUVB1, RUVB2, RPAP3, PDRG1, UXT, P1H1D1, PFD2, PFD6, WDR92, RMP | X = 0.45 | RUVB1, RUVB2 | X = 0.75 |
| **Integrator** | - | - | - | - |
| **SWI/SNF** | CCT2, CCT3 | X = 0.13 | CCT3 | X = 0.11 |
| **NuRD** | PSMC1, PSMC2, PSMC3, PSMC4, PSMC5, PSMC6, PSMC7, PSMC8, PSMC9, PSMC10, PSMC11, PSMC12, PSMC13, PSMC14 | X = 0.16 | PSMC1, PSMC2, PSMC3, PSMC4, PSMC5, PSMC6, PSMC7, PSMC8, PSMC9, PSMC10, PSMC11, PSMC12, PSMC13, PSMC14 | X = 0.18 |
| **T-complex** | KARS, SCYE1, IARS, RARS, EPRS | X = 0.25 | - | - |
| **26S proteasome** | RPL0, RPL1, RPL3, RPL4, RPL5, RPL6, RPL7, RPL8, RPL9, RPL10, RPL11, RPL12, RPL13, RPL14, RPL15, RPL16, RPL17, RPL18, RPL19, RPL20 | X = 0.75 | RPL0, RPL1, RPL2, RPL3, RPL4, RPL5, RPL6, RPL7, RPL8, RPL9, RPL10, RPL11, RPL12, RPL13, RPL14, RPL15, RPL16, RPL17, RPL18, RPL19, RPL20 | X = 1.20 |
| **Uncharacterized** | TFG, FAM103A1, MRT4, ZNF326, ABCF2, ATPBD1C, KIAA0020 | 1.39, 0.47, 0.46, 0.43, 0.31, 0.28, 0.26 | TFG | 1.25 |
from chromatin during mitosis, we found a highly significant enrichment for GO processes directly linked to transcription or downstream of transcription, namely, RNA splicing, processing, export, and translation. An association between mitotic RNAPII and SR proteins or Sm snRNPs had previously been reported (10). In contrast, DNA-dependent processes that are largely independent from transcription (repair, replication) and such processes that may be considered to occur “upstream” from transcription (transcription factor activity, chromatin, chromatin modification) were less or not enriched. These analyses suggest that RNAPII interactions in mitosis are linked to the transcriptional activity of RNAPII in interphase, albeit independent of chromatin or ongoing transcription.

Our investigation of the GO term “translation” is due to the presence of ribosomal proteins, mostly identified as more abundant relative to the control antibody. Although the large number of cellular ribosomes (~10 million per HeLa cell; (39)) makes ribosomal proteins likely candidates for nonspecific interactors, the higher apparent abundance of translation-related proteins in the 8WG16 sample compared with the control antibody sample, and their identification above background in other RNAPII purifications (31, 33) or phosphoCTD-interactor purifications (40) may reflect an association between RNAPII and ribosomes previously reported during interphase (41, 42). In comparison, cytoskeletal proteins, an important class of contaminant proteins identified in a recent study as binding directly to commonly used immunoprecipitation matrices (43), were not enriched in our specific immunoprecipitations, but were instead enriched in our control immunoprecipitations (see Supplementary Data).

To investigate whether RNAPII interacts with proteins involved in mitotic processes, we also analyzed the presence of proteins annotated with mitosis-related GO terms. The number of such proteins in the total lysate sample is low (1–5 proteins) and their enrichment was not statistically significant (p values: 0.12–0.56). Equivalent results were obtained for fractions 2 and 8. RNAPII interactions with proteins involved in mitosis are therefore not prevalent.

**Phosphorylation of Mitotic RNAPII is Not Required for the Maintenance of Protein Interactions**—To investigate whether the identification of RNAPII interactors was influenced by the alkaline phosphatase (AP) treatment used to maximize immunoprecipitation efficiency with 8WG16, we analyzed a total lysate sample prepared in the absence of AP treatment and presence of phosphatase inhibitors. Although many RNAPII interactions identified in the context of co-transcriptional processes, such as RNA processing, are thought to be dependent on CTD phosphorylation, we identified only 13 additional proteins that had not been identified in the AP-treated sample (Figs. 2D, 2E). Several of these proteins are functionally linked with each other within the context of transcriptional elongation, a part of the transcription cycle in which the RPB1 subunit of RNAPII is highly phosphorylated, or are known to
interact with each other (2): the TFIIF subunits GTF2F1 (8 peptides) and GTF2F2 (6 peptides), the CTD phosphatase FCP1 (11 peptides), as well as CDC73 (2 peptides), a subunit of the PAF complex. Two other proteins with roles in transcriptional elongation appear in both /H11001 AP and –AP lysates, but are more abundant in the –AP sample: the DSIF subunit SPT5H (8 and 1 peptides, respectively) and the TFIID subunit TAF9 (3 and 1 peptides, respectively). The small overall difference between the mass-spectrometric results of the /H11001 AP and –AP samples (Figs. 2D,2E) suggests that the maintenance of the association of most proteins with mitotic RNAPII is independent of the phosphorylation state of RPB1 and its associated proteins, although phosphorylation may be important for recruitment. The presence of hypophosphorylated RNAPII (RNAPIIA) in early gel filtration fractions (Fig. 1D) is also consistent with phosphorylation-independent interactions between RNAPII and associated proteins within the larger size complexes.

**Meta-analysis of RNAPII Mass-Spectrometric Data—**To compare our data for mitotic RNAPII interactors to that of three recent studies investigating RNAPII associated proteins in cycling human cells (31–33), we determined the degree of overlap between nonredundant lists of the proteins identified in the four studies. In brief, Das et al. (2007) analyzed RNAPII

| Name | Identifier | PAI Lysate | Fraction 2 | Fraction 8 | ΣPAI |
|------|------------|-----------|------------|------------|------|
| RPB5 | IPI00291093 | 1.00      | 0.62       | 0.69       | 2.31 |
| RPB7 | IPI00218895 | 1.20      | 0.50       | 0.50       | 2.20 |
| RPB4 | IPI00007283 | 1.00      | 0.50       | 0.50       | 2.00 |
| RPB8 | IPI0003309  | 0.60      | 0.60       | 0.80       | 2.00 |
| TFG  | IPI00294619 | 1.33      | (0.07)     | 0.60       | 2.00 |
| RPB3 | IPI00182286 | 0.88      | 0.44       | 0.50       | 1.81 |
| RPB10| IPI00003319 | 1.00      | 0.40       | 0.40       | 1.80 |
| RPB2 | IPI0027808  | 1.07      | 0.35       | 0.36       | 1.78 |
| RPB11| IPI00873238 | 1.14      | 0.29       | 0.29       | 1.71 |
| RPB1 | IPI00031627 | 1.05      | 0.36       | 0.27       | 1.68 |
| RPB9 | IPI00006113 | 0.89      | 0.33       | 0.44       | 1.67 |
| RPB6 | IPI00015119 | 1.00      | (0.20)     | 0.40       | 1.60 |
| ILF2 | IPI00005198 | 0.67      | 0.71       | 0.10       | 1.48 |
| RUVB1| IPI00021187 | 0.71      | 0.38       | 0.25       | 1.33 |
| RUVB2| IPI0009104  | 0.79      | 0.28       | 0.21       | 1.28 |
| GDOWN1 (GRINL1A) | IPI00552141 | 0.76      | 0.24       | 0.28       | 1.28 |
| EF1A1| IPI00396485 | 0.28      | 0.60       | 0.32       | 1.20 |
| FUS  | IPI00260715 | 0.71      | 0.12       | (0.06)     | 0.88 |
| RAPAP2| IPI00293375 | 0.69      | 0.05       | 0.12       | 0.86 |
| HNRPK| IPI00216746 | 0.52      | 0.26       | (0.04)     | 0.81 |
| NONO | IPI00304596 | 0.42      | 0.23       | 0.12       | 0.77 |
| RBP56| IPI00873762 | 0.18      | 0.15       | 0.13       | 0.45 |
| HNRPD| IPI00028888 | 0.27      | (0.07)     | (0.07)     | 0.40 |
| PRP19| IPI00004968 | 0.18      | 0.14       | (0.05)     | 0.36 |
| SERPING1| IPI00879531 | 0.07      | 0.11       | 0.11       | 0.29 |
| EWS  | IPI00098451 | 0.09      | 0.09       | 0.09       | 0.26 |
| C1R  | IPI00296165 | 0.11      | 0.07       | 0.07       | 0.24 |
| C1QB | IPI00477992 | (0.08)    | (0.08)     | (0.08)     | 0.23 |
| NAP1L1| IPI00023860 | (0.07)    | (0.07)     | (0.07)     | 0.21 |
| TIF1B| IPI00438229 | 0.06      | (0.03)     | 0.06       | 0.14 |
| CP   | IPI00017601 | (0.02)    | (0.02)     | 0.06       | 0.10 |
| C4B  | IPI00889723 | 0.02      | (0.01)     | 0.02       | 0.06 |

Meta-analysis of RNAPII Mass-Spectrometric Data—To compare our data for mitotic RNAPII interactors to that of three recent studies investigating RNAPII associated proteins in cycling human cells (31–33), we determined the degree of overlap between nonredundant lists of the proteins identified in the four studies. In brief, Das et al. (2007) analyzed RNAPII

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**Fig. 2.** Analysis of RNAPII-associated proteins identified by mass-spectrometry. A, Western blots against the URI/prefoldin subunits RPAP3 and RUVB2 and against TFG, ACTL8, QSER1 and ALMS1, confirm respective mass-spectrometric detection (asterisks) or absence in the total lysate, fraction 2 and fraction 8. B, Proportional Venn diagram comparing RNAPII-associated proteins among total lysate and fractions 2 and 8. C, GO analysis for proteins identified in the total lysate, fraction 2 and fraction 8 reveals very strong enrichment for GO terms associated with transcription and downstream processes. D, Proportional Venn diagram comparing the proteins identified in RNAPII complexes treated ±AP, and lists of proteins specific to –AP or +AP. Overlap includes proteins present in both samples that were specific (identified with ≥2 peptides) or more abundant in only one of the two samples. E, Comparison of peptide abundance indices in total lysates ±AP, revealing that phosphorylation of RPB1 has little contribution to the relative abundance of proteins in the two samples (Pearson product-moment correlation coefficient of 0.897). See supplementary Fig. S2 for evidence that antibody 8WG16 can immunoprecipitate RPB1 phosphorylated on Ser2 and/or Ser5 of the CTD. F, Venn diagram comparing the RNAPII associated proteome identified in the present study to three previous proteomic analyses of RNAPII. For Jeronimo et al. (2007), only proteins directly co-immunoprecipitated with RNAPII subunits were included.
complexes from a HeLa cell nuclear extract after 8WG16 immunoprecipitation and total elution with gel loading buffer, Jeronimo et al. (2007) from EcR-293 whole cell extracts after immunoprecipitation of TAP-tagged RNAPII subunits, and McCracken et al. (2005) from a HeLa cell nuclear extract after 8WG16 immunoprecipitation and high salt elution.

The meta-analysis revealed a set of proteins exclusive to each purification stratagem (Fig. 2F, supplementary Table S3), with 333 proteins specific to the present study, as compared with 30, 25, and 16 for the McCracken et al., Das et al. and Jeronimo et al. data sets, respectively. Of note, among the known RNAPII-associated proteins exclusively identified in our study, many have high PAI values (e.g. SMN, NONO, PRMT1), showing that the differences in protein detection between the present and previous studies are not simply due to higher detection sensitivity of our mass spectrometry method, but most likely reflect inherent differences in the purification strategies.

Our proteomic analyses of RNAPII complexes, extracted in mild conditions from mitotic extracts, identify novel RNAPII-associated proteins, including previously characterized (e.g. ALMS1, DBC1), and uncharacterized proteins (e.g. FAM103A1, ABCF2, ZNF326, QSER1). GO analysis of the 333 RNAPII interactors exclusively identified in our study revealed enrichment for various terms associated with transcription and related processes (Fig. 2F).

No proteins were identified as common to all four studies, although several proteins, mostly involved in splicing and other transcription related processes, were found in at least three of the four studies. Several proteins co-purified with RNAPII in two of the studies, including proteins involved in translation.

**RNAPII Interactors Colocalize With RNAPII During Mitosis—**To determine whether the RNAPII-associated proteins identified in vitro also associate with RNAPII in situ, we performed antibody-blocking assays as a high-resolution method to determine colocalization between candidate proteins and RNAPII (20, 41, 44). Antibody-blocking assays measure the decrease in binding of one antibody after pre-incubation with a test antibody; if the two target epitopes lie within the size range of the antibody molecules used to detect them (~9 nm in the case of IgG type antibodies), binding of the first (blocking) antibody produces steric hindrance that prevents binding of the second (test) antibody, resulting in decreased fluorescence intensity.

We performed antibody-blocking assays as previously shown using ultrathin (~150 nm thick) cryosections of fixed cells (20, 44), which optimally preserve RNAPII organization and avoid extraction of relevant nuclear proteins (45). Briefly, cryosections were first immunostained using a set of available antibodies against proteins of interest or a control antibody raised against biotin that specifically detects mitochondria in the cytoplasm, before being immunostained using 8WG16 (20). To measure the colocalization of candidate interactors with RNAPII independently of its phosphorylation state, cryosections were pretreated with alkaline phosphatase to maximize 8WG16 detection of RNAPII (19; data not shown). Images from mitotic cells were collected in the same conditions for control and antibody-blocked sections. We analyzed both known (RUVB1, RUVB2, ILF2, TFG) and novel (QSER1, ALMS1, RPS6, RPL26) RNAPII interactors. A positive control antibody detecting Sm proteins, known to associate with RNAPII, was also employed (19). To investigate whether RNAPII interactors identified in HeLa cells also associate with RNAPII in primary human cells, we performed antibody-blocking experiments in both HeLa cells and human primary T lymphocytes (PHA-activated peripheral blood mononuclear cells).

As expected, the antibody-blocking experiments showed colocalisation between the CTD of RPB1 and RPB2 in both HeLa cells and lymphocytes (Fig. 3). Furthermore, statistical analysis showed significant decreases in the staining intensity of 8WG16 after incubation with antibodies to RNAPII-interactors TFG, QSER1, and ALMS1, as well as with the positive controls RPB2 and Sm (all in comparison with antibiotin), demonstrating their close proximity to the CTD of RPB1 in mitotic HeLa cells and lymphocytes (Figs. 3A, 3B). ILF2 and RUVB2 blocking effects were only statistically significant in HeLa cells (p < 0.001 and p < 0.05, respectively). The colocalization of RNAPII interactors in both HeLa cells and primary lymphocytes shows that their association with RNAPII is unlikely to be cell-type or cancer specific. It is worthwhile noting that lack of colocalization in this assay does not exclude the possibility that proteins interact with RNAPII in a complex, if the respective epitopes tested lie more than 10 nm apart, for example if they interact with the RNAPII complex further away from the CTD (detected by 8WG16), or if their association with RNAPII is of an indirect nature.

**Interactor Requirement for Transcript Elongation During Interphase—**Our strategy to identify RNAPII-associated proteins uses mitotic cell extracts. To investigate whether the novel RNAPII interactors have functions in the transcriptional activity of RNAPII complexes during interphase, we conducted a small-scale functional screen to determine changes in levels of ongoing transcription and nuclear organization at the single-cell level.

We transfected HeLa cells with siRNAs against 25 RNAPII interactors and employed an *in situ* transcription assay based on the incorporation of 5-bromouridine 5′-triphosphate (Br-UTP) by engaged RNAPII complexes (18). For this analysis, we chose proteins (1) previously known to associate with RNAPII (RUVB1, RUVB2, MATR3, GDOWN1, RPAP3, RPAP2, ILF2, NUMA1, HNRPD, XAB1/MRN1, TFG), (2) exclusively identified in our study as RNAPII associated, but which have either been previously characterized (CCT3, WDR92, MYBBP1A/MBB1A, ALMS1, FAM120A/F120A), or (3) that were completely uncharacterized (QSER1, C20ORF77/RPRD1B, KIAA0460/RPRD2, ATPBD1C/GPN3, FAM103A1/F103A, ABCF2, ACTL8, C1ORF77/CAN77, ZNF326).
The levels of transcript elongation measured for each knockdown after immunofluorescence detection of Br-labeled RNA were compared with levels obtained with negative control siRNA. Treatments with siRNAs that lead to a knockdown of the RNAPII subunits RPB2 and RPB4 resulted in roughly 60–70% of the levels of newly-made transcripts ob-

**Fig. 3.** Antibody-blocking assay reveals in situ colocalisation between RPB1 and several candidate proteins in mitosis. A, Cryosections were first treated with AP to maximize 8WG16 binding to total RPB1, and indirectly immunolabeled in the absence (negative control) or presence of antibodies to candidate proteins (QSER1 or ALMS1), positive blocking antibody against RPB2, or an unrelated anti-biotin antibody that detects mitochondria in the cytoplasm (arrowhead, negative control). After cross-linking of the first immunocomplex, sections were indirectly immunolabeled with 8WG16 (red). Nucleic acids were counterstained with DAPI (blue), and confocal images collected using the same settings without signal saturation. Scale bar: 6 μm. Pre-incubation with anti-RPB2 reduces the intensity of 8WG16 (RPB1) signal throughout the nucleoplasm. Control pre-incubation with antibiotin control antibody before labeling with 8WG16 has little or no effect. B, Antibody inhibition assays (ABA) in mitotic cells show a decrease in 8WG16 detection after blocking with antibodies against RPB2, TFG, QSER1, ALMS1, and Sm proteins, in both HeLa cells and primary human PHA-activated peripheral blood mononuclear cells. In HeLa cells, colocalisation is also detectable after blocking with antibodies against RUVB2 and ILF2. Asterisks denote statistical significance relative to the anti-biotin sample at significance levels of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (**). Average and SD of two coverslips are represented. Number of nuclear profiles was ≥5 for each coverslip. Statistical significance was tested using ANOVA and Benjamini-Hochberg correction for multiple testing; uncorrected $p$ values are indicated in supplementary Table S5.
Many RNAPII interactors identified in mitosis are functionally and cytologically associated with RNAPII during interphase. A, Br-UTP transcription assay after siRNA-mediated knockdown of candidate proteins. Representative images for Br-RNA labeling after RNAi against a selection of targets. Scale bar: 10 μm. Graph represents mean nuclear Br-RNA intensities per cell (average and SD from two
tained with control siRNA (Figs. 4A, 4B). Greater effects were likely not observed because of incomplete depletion of RNAPII subunits, despite good transcript depletion detected by qRT-PCR of total RNA (Fig. 4A), or due to the death of more severely affected cells, presence of untransfected cells, and background transcription by RNAPIII. Pre-incubation of permeabilized HeLa cells with a concentration of the transcription inhibitor α-amanitin (1 μg/ml) that completely inhibits transcription by RNAPII, but which leaves RNAPIII unaffected, yields levels of Br-RNA detection of 7–9% of the levels observed in untreated cells (supplementary Fig. S3), as expected (18).

We find that 14 of the 25 RNAPII interactors tested by siRNA knockdown are involved in sustaining normal levels of transcript elongation in interphase (Fig. 4A), including six out of 13 proteins that were exclusively identified as RNAPII interactors in this study. Interestingly, we identify proteins that are required to support transcriptional activity (RUVB1, RUVB2, HNRPD, QSER1, ALMS1, TFG, ILF2, FAM120A, ACTL8), as well as proteins that have an inhibitory effect on levels of transcription elongation (XAB1, MYBBP1A, RPAP2, C20orf77, FAM103A1), as shown by an increase in transcriptional activity after RNAi. Knockdown of target mRNAs was confirmed by qPCR for all candidate proteins (Fig. 4A, supplementary Table S4). Two proteins, which were also identified by Jeronimo et al. to be associated with nuclear RNAPII complexes, RPAP2 and XAB1, were shown here to be functionally relevant to the activity of RNAPII, with knockdown of either of these proteins leading to an increase in transcriptional activity. A yeast homolog of RPAP2, Rtr1, has recently been identified as a potential RNAPII-S5p specific phosphatase (46). In those cases in which RNAi knockdown does not affect elongation, candidate proteins may be involved in other aspects of transcription, or levels of protein knockdown may be insufficient to reveal a phenotype, depending on the stability of each protein studied.

Among the proteins that colocalize with RNAPII during mitosis are several with a clear involvement in nuclear transcription (QSER1, ALMS1, ILF2, RUVB2, TFG; Figs. 3B, 4A). We first asked whether several of these RNAPII interactors were localized inside the nucleus during interphase. Importantly, all tested antibodies against candidate RNAPII interactors led to detectable staining within the cell nucleus (Fig. 4B and data not shown). This finding is especially significant for ALMS1, a protein defective in Alström syndrome. Although ALMS1 contains nuclear localization signals and a putative leucine zipper (47, 48), previous studies reported its localization to centrosomes and the basal bodies of cilia (49). Nuclear localization of ALMS1 was confirmed using two independent antibodies, raised against epitopes in the internal part of the protein or the C-terminal region. The two antibodies also show nuclear and cytoplasmic staining in cryosections of murine pancreatic and hypothalamic tissue samples (Fig. 4B), both of which are associated with Alström syndrome pathology (50). Immunolocalisation in ultrathin cryosections (∼150 nm thickness) optimally preserves nuclear architecture and the localization of nuclear proteins such as TBP and RNAPII (45), raising the possibility that the nuclear localization of ALMS1 might not have been detected in previous studies because of the cellular preparation before immunolabelling, which could have hindered antibody accessibility or resulted in protein extraction.

To test whether the physical association between RNAPII interactors identified in mitosis is retained during interphase, we repeated the antibody-blocking experiment using interphase cells of both primary PHA activated peripheral lymphocytes and HeLa cells. The blocking experiments reveal that the colocalization and physical association with RNAPII observed for the proteins RUVB2, ILF2, TFG, QSER1, and ALMS1 during mitosis are retained during interphase, both in HeLa cells and in primary lymphocytes (Fig. 4C), thus showing that the effects on global transcription observed for these proteins are likely of a direct nature.

In summary, the results of the transcription and colocalization assays serve to validate the low stringency, mitosis-based approach to identifying proteins that functionally interact with RNAPII in both mitosis and interphase.

**DISCUSSION**

We set out to isolate RNAPII and its associated proteins from mitotic cells as a strategy to circumvent the harsh extraction conditions required for nuclear yields levels of Br-RNA detection of 7–9% of the levels observed in untreated cells (supplementary Fig. S3). Two independent experiments are shown per candidate protein. Knockdowns of RPB2 and RPB4 served as positive controls. Transcriptional activity is expressed relative to matched AllStars Negative siRNA control (set to 100%). Asterisks mark statistical significance relative to control siRNA at significance levels of \( p < 0.05 \) (*), \( p < 0.01 \) (**), and \( p < 0.001 \) (***)]. Statistical significance was tested using ANOVA and Benjamini-Hochberg correction for multiple testing. See supplementary Fig. S3 for images from the remaining treatments and a different graphical representation of the results. B, Immunolabelling of cryosections demonstrates nuclear localization of QSER1, TFG and ALMS1 in HeLa cells. Nuclear localization of ALMS1 was also tested in murine pancreatic and hypothalamic tissue, using antibodies directed against internal or C-terminal epitopes of human ALMS1. Nucleic acids were counterstained with DAPI (not shown); nuclear perimeters are represented as white dotted lines. Scale bars: 6 μm. C, Antibody inhibition assay (ABA) in interphase cells shows colocalisation between RBP1 and associated proteins identified in mitoses. Measurement of average 8WG16 intensity across the nucleoplasm in both HeLa cells and human PHA-activated peripheral blood mononuclear cells show a decrease in 8WG16 detection after blocking with antibodies against RPB2, TFG, QSER1, ALMS1, RUVB2, ILF2, and Sm proteins. Asterisks denote statistical significance relative to the anti-biotin sample at significance levels of \( p < 0.05 \) (*), \( p < 0.01 \) (**), and \( p < 0.001 \) (***)]. Error bars: SD of two coverslips. Number of nuclear profiles was ≥5 for each coverslip. Statistical significance was tested using ANOVA and Benjamini-Hochberg correction for multiple testing; uncorrected \( p \) values are indicated in supplementary Table S5.
traction conditions usually employed in purifications of RNAPII from unsynchronized, largely interphasic cells. We combined our isolation method with control immunoprecipitations, a specific elution regimen, and highly sensitive mass spectrometry, leading to the identification of a large number of novel and known interactors. Validation of RNAPII associations by functional and cytological methods for several candidate proteins confirms a significant proportion of novel interactors.

In line with earlier reports, we find that mitotic RNAPII is associated with factors involved in RNA splicing and processing (10), and is phosphorylated on the S2 and/or S5 residues of the RPB1 C-terminal domain (51). However, the demonstration that phosphorylation is restricted to the subpopulation of larger RNAPII complexes (Fig. 1D) may suggest a direct relationship between these two observations. In light of the generally held view of recruitment of RNA processing factors by phosphorylated RNAPII complexes during transcription, association of RNA processing factors with RNAPII during mitosis may also directly result from RNAPII phosphorylation, and serve to facilitate the co-ordinated postmitotic nuclear re-import. However, some of the associations observed between RNAPII and RNA processing factors during mitosis, in light of their insensitivity to RNAPII dephosphorylation, are indicative of more permanent interactions.

A group of 32 proteins was consistently identified in the three samples treated with alkaline phosphatase. The presence of these proteins in all of these samples suggests that they bind to RPB1 directly or indirectly by interactions among each other. Most of these proteins (25 out of 32) have previously been implicated in transcription-related processes. These include recently identified RNAPII associated proteins such as GDOWN1 and RPAP2, and proteins that so far had only been implicated in the transcription of specific genes (ILF2, RUVB1, RUVB2). SiRNA knockdown demonstrated a global requirement for ILF2 and RUVB1/2 in transcriptional elongation, as well as for MYBBP1A, which was exclusively found in the prefractionation lysate and has previously been shown to be important for the transcription of selected genes (52). RUVB1 and RUVB2 have been identified as constituents of a variety of protein complexes (53). It will be interesting to see whether they perform comparable functions in all of these complexes, be it a role in complex assembly, DNA binding, or other processes.

Especially noteworthy is the identification of three previously uncharacterized proteins as functional interactors of RNAPII: QSER1, C20ORF77, and ACTL8. Our results imply a role for these proteins within the context of transcription, as they copurify with RNAPII in mitotic cells and support the normal transcriptional activity of RNAPII in interphase. For QSER1, we demonstrated colocalization with RNAPII in both interphase and mitosis (Figs. 3B, 4C); for ACTL8, the available antibodies did not stain cryosections, whereas for C20ORF77 preliminary antibody-blocking experiments also show some level of blocking (not shown). C20ORF77 appears to be orthologous to yeast Rtt103 and C. elegans CIDS-1, proteins shown to be involved in transcriptional termination (54, 55). An involvement of C20ORF77 in transcription termination is in agreement with the observed increase in Br-UTP incorporation during transcript elongation upon knockdown of the protein.

Importantly, we have also functionally verified two novel interactors of RNAPII that are involved in human disease. The gene coding for ALMS1 is mutated in patients suffering from Alström syndrome (47, 48), a progressive and multisystemic disorder that is characterized by neurosensory, developmental, and metabolic dysfunctions (50), including diabetes and obesity. The ALMS1 protein has previously been shown to localize to centrosomes and to the basal body of cilia (49), a fact that fits well with some of the pathology observed in Alström syndrome. In our study, we find that ALMS1 co-purifies with RNAPII, and that it is important for sustaining the transcriptional activity of RNAPII. We also find that it localizes in the interphase nucleus in both human cells and murine tissues, where it lies in close proximity to RNAPII, as shown by antibody-blocking assays. This data implies that the molecular basis of Alström syndrome is more complicated than has been previously appreciated, involving control of gene expression, and may lead to a better understanding of the dysfunctions observed in this syndrome.

The second verified RNAPII interactor involved in disease formation is TFG, which forms oncogenic fusion proteins in various cancer types involving either tyrosine kinases or the transcription factor NOR1 (56). TFG contains a SYGQ-rich region that shares similarity with the SYGQ-rich regions of the TET family of proteins, TLS/FUS, EWS, and TAF15/RBP56, all of which co-purify with RNAPII in all of our samples. The members of the TET family are known to associate with RNAPII and to take part in transcription-related processes (57), and may have roles in motor neuron disease (58). They also form oncogenic fusion proteins with transcription factors in which their SYGQ-rich regions are fused to the DNA-binding domains of their fusion partners. The SYGQ-rich regions are thought to act as transactivation domains in these fusion proteins. The co-purification of TFG with RNAPII, its effect on transcriptional activity, and colocalization with RNAPII suggest that the pathology of the TFG-NOR1 fusion protein is likely based on its ability to transactivate RNAPII.

The large number of RNAPII interactors found associated with native complexes easily isolated from the mitotic cytoplasm raises the possibility that other protein complexes involved in chromatin-associated processes may, at least partially, dissociate from mitotic chromosomes intact. Therefore, our approach of using mitotic lysates, as starting materials for the isolation of protein complexes and their associated factors, may be more widely applicable to other nuclear complexes, and may lead to the identification of proteins otherwise lost due to the unphysiological purification conditions often required to extract nuclear proteins.
In summary, using mild lysis conditions in the preparation of RNAPII complexes from mitotic cells we have significantly expanded the proteome of RNAPII associated proteins, providing an important resource for understanding RNAPII function and regulation in humans. We also show that several previously uncharacterized proteins and two proteins involved in disease formation in humans are important for the transcriptional activity of RNAPII, thus opening the door for more detailed investigations into their functions.

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††† This article contains supplemental Figs. S1 to S3 and Tables S1 to S5.

To whom correspondence should be addressed: MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK. Tel.: +44.2083832323; Fax: +44.2083833036; E-mail: andre.moeller.mrc@imperial.ac.uk.

‡‡‡ Present address: Department of Biochemistry and Molecular Biophysics, Columbia University Medical Center, New York, NY 10032, USA.

†† Present address: Tenovus Laboratory, Cancer Sciences Division, Southampton University School of Medicine, General Hospital, Southampton, United Kingdom.

Author contributions: AM, designed and performed experiments, designed and performed data and bioinformatic analyses, wrote paper; SQX, designed and performed experiments and performed data analyses (protein immunofluorescence localization and antibody blocking); FH, performed mass-spec runs and processed raw mass-spec data; BL, designed and performed bioinformatic analyses (GO, comparisons with other studies, other analyses with negative results); HP, designed and assisted in initial mitotic extract preparation, gel filtration and western blotting studies; SM, characterized initial gel filtration fractions by western blotting; FR, performed ANOVA for gel filtration and western blotting studies; SM, characterized initial gel filtration fractions by western blotting; HP, designed and assisted in initial mitotic extract preparation, gel filtration and western blotting studies; MS, designed and performed mass-spec runs and processed raw mass-spec data; AP, designed and performed initial mitotic extract preparation, gel filtration and western blotting studies, designed experiments and bioinformatic studies, wrote paper.

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