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The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1

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Activation of gene transcription in metazoans is a multistep process that is triggered by factors that recognize transcriptional enhancer sites in DNA. These factors work with co-activators to direct transcriptional initiation by the RNA polymerase II apparatus[1]. One class of co-activator, the TAF_{II} subunits of transcription factor TFIID, can serve as targets of activators and as proteins that recognize core promoter sequences necessary for transcription initiation[2–5]. Transcriptional activation by enhancer-binding factors such as Sp1 (ref. 6) requires TFIID, but the identity of other necessary cofactors has remained unknown. Here we describe a new human factor, CRSP, that is required together with the TAF_{II}s for transcriptional activation by Sp1. Purification of CRSP identifies a complex of approximate relative molecular mass 700,000 (M_r ~700K) that contains nine subunits with M_r values ranging from 33K to 200K. Cloning of genes encoding CRSP subunits reveals that CRSP33 is a homologue of the yeast mediator subunit Med7 (ref. 7), whereas CRSP150 contains a domain conserved in yeast mediator subunit Rgr1 (ref. 8). CRSP p200 is identical to the nuclear hormone-receptor co-activator subunit TRIP2/PBP[9,10]. CRSPs 34, 77 and 130 are new proteins, but the amino terminus of CRSP70 is homologous to elongation factor TFIIS[11]. Immunodepletion studies confirm that these subunits have an essential cofactor function. The presence of common subunits in distinct cofactor complexes suggests a combinatorial mechanism of co-activator assembly during transcriptional activation.

Sp1 is important in the transcriptional regulation of genes from *Drosophila* to humans. Previous studies showed that the interaction of the glutamine-rich activation domains of Sp1 with the TAF_{II} subunits of TFIID (where TAF is TATA-binding protein (TBP)-associated factor) is required for *in vitro* transcriptional activation[4]. However, these early studies were done using a partially purified transcription system and did not address the question of whether another cofactor(s) may be necessary to potentiate activation by Sp1 fully. Other cofactor fractions may indeed enhance activator-dependent transcription *in vitro*[12]. One such crude fraction (USA) is known to contain many positive and negative cofactors, including PC4, a single-stranded DNA-binding protein, and PC2, a complex of M_r >500K, whose contribution to transcription activation by Sp1 has not been fully defined[13–15]. We therefore set out to characterize the cofactors that are required for activation by Sp1.

To identify potential Sp1 cofactors, we first developed an *in vitro* transcription assay that consisted of purified recombinant TFIIA, partially purified RNA polymerase II and basal transcription factors TFIIB, E, F and H, but contained no additional cofactor fraction such as USA. This mixture was supplemented with either antibody-affinity purified TFIID or recombinant TBP. As expected, reactions lacking TFIID or TBP (Fig. 1a) failed to support any detectable transcription from either the test template containing three GC-box Sp1-recognition sites or a control template lacking Sp1-binding sites. Thus, none of the partially purified general transcription factors is substantially contaminated with endogenous TFIID. When we added immunopurified human TFIID to the transcription system, we detected basal levels of transcription from both templates but little Sp1-directed activation (Fig. 1a). By contrast, when we added a phosphocellulose fraction (P1M) containing endogenous TFIID and several putative cofactor activities (including USA)

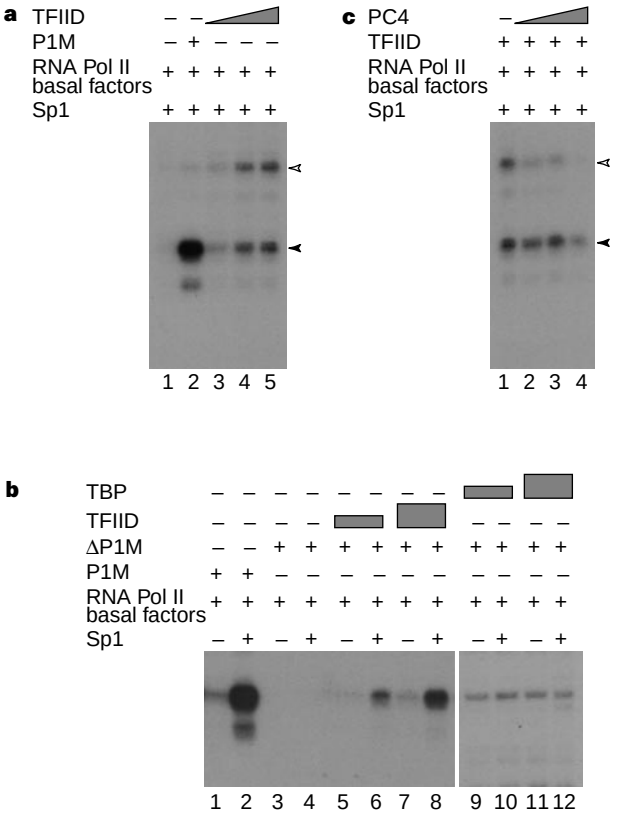

Figure 1 Transcriptional activation by Sp1 requires a new cofactor. **a**, Factor requirements for Sp1-dependent activation. Transcription reactions contain Sp1 (lanes 1–5), RNA polymerase II and basal transcription factors (lanes 1–5), the 1 M phosphocellulose fraction (P1M; lane 2) and increasing amounts of immunopurified TFIID (lanes 3–5). Transcription from a GC-box-containing template and a control DNA template lacking Sp1-binding sites is indicated by filled and open arrowheads, respectively. **b**, TFIID and a cofactor in the P1M fraction are needed to potentiate Sp1-dependent activation. Transcription reactions contain the GC-box template (lanes 1–12), Sp1 (lanes 2, 4, 6, 8, 10 and 12), RNA polymerase II and basal transcription factors (lanes 1–12), the P1M fraction (lanes 1 and 2), the P1M fraction treated with anti-TBP affinity resin to deplete TFIID (ΔP1M, lanes 3–12) and increasing amounts of immunopurified TFIID (lanes 5–8) or TBP (lanes 9–12). **c**, PC4 cannot substitute for the cofactor(s) present in the ΔP1M fraction. Transcription reactions were performed as described in **a**, in the presence of increasing amounts of PC4 (lanes 2–4) instead of the ΔP1M or P1M fraction.

to these reactions, we observed strong activation (20-fold) of transcription from the GC-box-containing template but not from the control template. The P1M fraction therefore contains one or more cofactors required for activation by Sp1.

To uncover new cofactor activities in the TFIID-containing P1M fraction, we had to remove contaminating TFIID. We efficiently depleted both TFIID and TBP from this fraction by multiple rounds of immunoprecipitation with anti-TBP antibodies. As expected, the resulting TFIID-free cofactor fraction no longer supported basal or activated transcription (Fig. 1b). The addition of immunopurified TFIID containing the full complement of TAF_{II} subunits together with the depleted cofactor fraction (ΔP1M) restored robust activation by Sp1 (Fig. 1b). Addition of purified recombinant TBP and the cofactor fraction restored basal transcription but not responsiveness to Sp1 (Fig. 1b). This indicates that efficient activation by Sp1 requires both TFIID and one or more cofactor activities present in the P1M fraction. Because the P1M fraction probably contains the positive cofactor PC4, we next tested the ability of purified recombinant PC4 to substitute for the P1M activity. PC4 inhibited basal transcription and was unable to support activation by Sp1 when added in combination with TFIID (Fig. 1c). At higher concentrations, PC4 appeared to inhibit transcription from both templates.

To characterize the polypeptide composition and biochemical properties of the new Sp1 cofactor, we purified the cofactor activity through five consecutive chromatographic columns and glycerol gradient sedimentation (Fig. 2a). After each chromatographic step, we identified the fractions containing the activity by assaying for Sp1-dependent activation (Fig. 2b) in the absence of added USA. We estimated that after MonoS anion-exchange chromatography, the specific activity of the purified cofactor was ~50,000–100,000-fold higher than in crude nuclear extracts. The highly purified fraction thus contained a cofactor required for Sp1 activation (CRSP) that is distinct from PC4. As CRSP can replace the USA fraction, it may represent one of the activities present in USA, such as PC2. However, because the identity of the polypeptide(s) representing PC2 activity has not been defined, we cannot yet ascertain the relationship between CRSP and PC2. As a preliminary biochemical characterization of CRSP, we performed both glycerol gradient sedimentation and gel-filtration analysis to assess the native relative molecular mass of this cofactor (data not shown). Our results indicate that CRSP behaves as a single complex with an apparent M_r value of 700K, consistent with it being a multisubunit cofactor.

Using nuclear extracts prepared from 400 litres of HeLa cells, we obtained ~20 μg of purified CRSP. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the most extensively purified MonoS fractions revealed a pattern of nine to ten polypeptides that consistently co-purified with CRSP transcriptional activity (Fig. 2c, d). The polypeptides that co-chromatographed with CRSP included species of 33K, 34K, 70K, 77K, 130K and 150K. Most of the preparations also contained polypeptides of 85K, 100K and 200K. In contrast, the 175K band failed to co-purify on a reproducible basis. We therefore tentatively identified nine polypeptides as putative CRSP subunits and targeted them for further analysis.

The MonoS-purified CRSP was subjected to preparative SDS–PAGE separation, followed by transfer to nitrocellulose membrane and extraction by LysC protease digestion of individual polypeptides. The proteolytic peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) and subjected

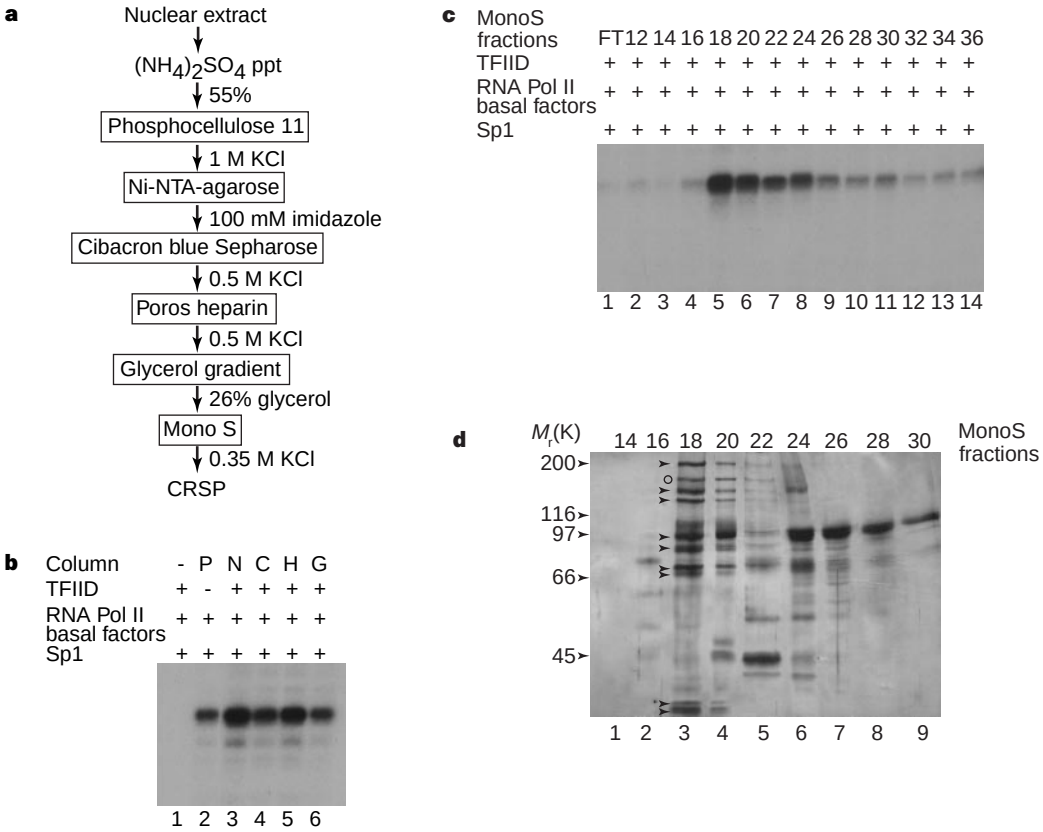

Figure 2 Purification of CRSP. **a**, Procedure for purification of CRSP. **b**, Reconstituted transcription reactions with the GC-box template, Sp1, RNA polymerase II and basal transcription factors (lanes 1–6) and immunopurified TFIID (lanes 1, 3–6) were supplemented with CRSP fractions recovered from phosphocellulose (P), Ni-NTA-agarose (N), Cibacron Blue Sepharose (C), Poros heparin (H) or glycerol gradient centrifugation (G). **c**, Transcription profile of CRSP activity after MonoS chromatography. Reactions were supplemented with the indicated MonoS fractions (lanes 1–14) or flow-through (FT) fraction. **d**, Silver-stained SDS–PAGE gel of the MonoS fractions. Arrowheads indicate the proteins that co-migrate with CRSP activity. The open circle indicates a protein that fails to co-purify with CRSP in a reproducible manner.

to microsequence peptide analysis. Multiple peptide sequences derived from seven subunits were used to generate probes and/or to identify expressed sequence tag (EST) sequences. Several probes obtained by this strategy were used to screen human complementary DNA libraries for sequences encoding the putative CRSP subunits. (See Supplementary Information for the amino-acid sequences of the identified subunits of CRSP.)

The sequences show that CRSP represents a new cofactor complex for Sp1 that shares some subunits with other cofactors but also contains some unique subunits. CRSP34 and CRSP130 encode new human proteins with no apparent homology to other known gene products. In contrast, both CRSP33 and CRSP150 share homology to components of the yeast mediator, a complex that can potentiate activated transcription in yeast[16]. CRSP33 is a human homologue of yeast Med7 and the CRSP33 sequence is identical to the recently reported sequence of the human MED7 protein[7]. CRSP150 is identical to EXLM1, a novel protein of unknown function[17]. Residues 92–164 of CRSP150 show significant similarity to a yeast mediator component, Rgr1 (Fig. 3)[8]. The homology between the two proteins outside this region is not significant. In addition, CRSP77 and CRSP150 contain peptide sequences identical to those described for the p78 and p110 subunits of a murine complex that contains subunits similar to those of the yeast mediator[18]. CRSP150 contains peptide sequences identical to those described for the p150 subunit of NAT, a recently identified human complex that appears negatively to regulate activated transcription[19]. Although both Med7 and Rgr1 have previously been associated with a mediator complex, the other prototypic 'mediator' subunits, such as Srb proteins, Gal11 and the other Med proteins, do not appear to be present in CRSP. Instead, proteolytic peptides of the p200 subunit of CRSP match peptides of the TRIP2/PBP/RB18A protein[9,10,20], which has also been described, more recently, as DRIP230 and TRAP220, a component of a large co-activator complex that may be involved in inducible activation by nuclear hormone receptors such as peroxisome proliferator-associated receptor-γ, retinoid-X receptor, vitamin D receptor and others[21,22]. In addition, the N-terminal domain of CRSP70 shows similarity to transcription elongation factor SII (Fig. 3)[11].

We used the cDNA clones encoding the CRSP subunits to generate partial or complete recombinant proteins. We then raised polyclonal antisera directed against CRSP subunits and used these antisera to characterize the biochemical and functional properties of CRSP. We attempted to generate antibodies against most of the CRSP subunits; however, only the antisera against CRSP33, CRSP130 and CRSP150 efficiently recognized denatured proteins on western blots. Furthermore, only anti-CRSP150 antibody was able to immunoprecipitate the endogenous complex. We therefore used antigen-affinity-purified anti-CRSP150 antibodies to determine which polypeptides form a stable CRSP complex and to test whether these polypeptides are responsible for CRSP's cofactor activity. Anti-CRSP150 antibodies co-immunoprecipitated a complex of nine to ten polypeptides from the partially purified Ni-NTA-agarose chromatographic fraction. The polypeptide composition of the immunopurified complex was nearly identical to that of the most pure MonoS fractions, consisting of CRSPs 33, 34, 70, 77, 85, 100, 130, 150 and 200 (Fig. 4a). In addition, western blot analysis (see Supplementary Information) confirmed that the immunoprecipitated complex contained CRSPs 33, 130 and 150. Most of these subunits appeared to be stably associated into a complex that survived high-salt (1 M) washes (Fig. 4a, lane 4). We also confirmed that at least CRSPs 33, 130 and 150 co-purified as a stable

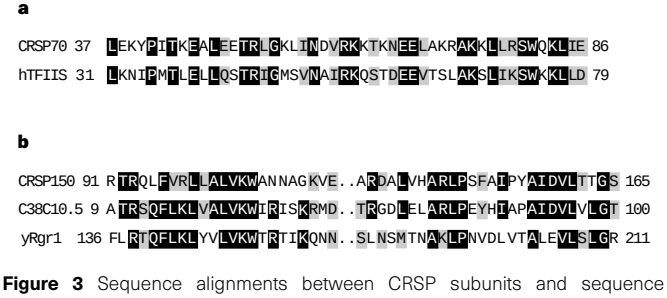

Figure 3 Sequence alignments between CRSP subunits and sequence homologues. **a**, The N terminus of CRSP70 shares sequence homology with the N terminus of the human transcription elongation factor IIS. **b**, The N-terminal region of CRSP150 shares significant sequence homology with the N-terminal region of the yeast mediator component Rgr1 and a novel *Caenorhabditis elegans* protein, C38C10.5. Black shading denotes identical residues; grey shading denotes similar residues.

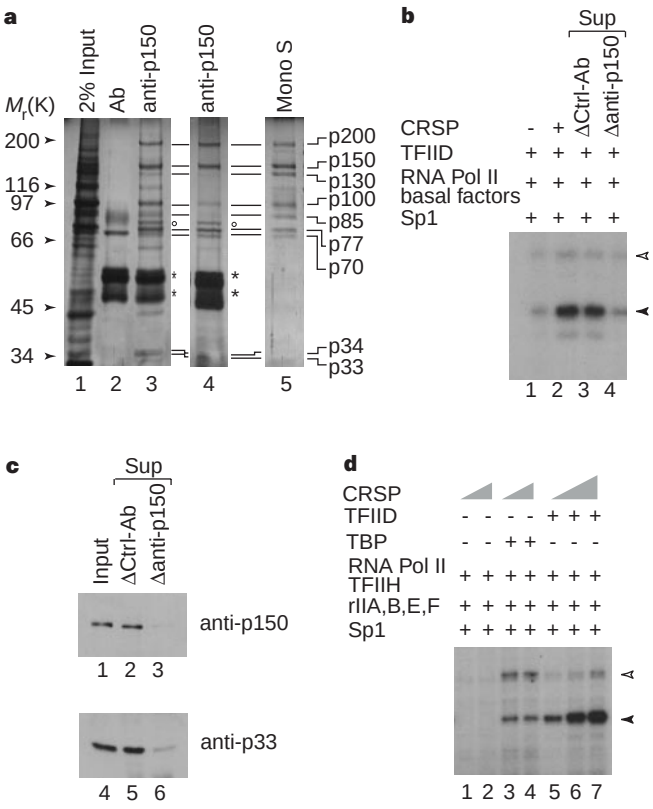

Figure 4 Immunoprecipitation of the CRSP complex and immunodepletion of CRSP activity. **a**, Comparison of polypeptides immunopurified with anti-p150 antibodies (lanes 3 and 4) to MonoS-purified CRSP (lane 5). Lanes 1 and 2 show the starting material for immunopurification and the antibody alone, respectively. The nine CRSP subunits are indicated by their respective apparent molecular weights. Asterisks denote the immunoglobulin heavy chain. Open circles indicate a protein that immunoprecipitated but is not present in the MonoS fraction. Lanes 3 and 4 show immunoprecipitation patterns after washes with 0.7 M and 1 M KCl, respectively. **b**, Immunodepletion of CRSP reduces transcription activated by Sp1. Transcription reactions were reconstituted as described (Fig. 1) and supplemented with a partially purified CRSP fraction (see Methods) (lane 2) or with the same fraction after immunodepletion with control antibodies (lane 3) or anti-p150 antibodies (lane 4). Transcription from a GC-box-containing template and a control template is indicated by the filled and open arrowheads, respectively. **c**, Western blots of the immunodepleted fractions produced using anti-p150 (lanes 1–3) and anti-p33 (lanes 4–6) antibodies. Lanes 1 and 4, the partially purified CRSP fraction (see **b**); lanes 2 and 5, supernatant of the same fraction after depletion with control antibodies; lanes 3 and 6, supernatant of the same fraction after depletion with anti-p150 antibodies. **d**, CRSP mediates Sp1-dependent activation in a highly purified transcription system. Transcription reactions were reconstituted with purified recombinant Sp1, recombinant TFIIA, B, E and F (rIIA, B, E, F), RNA polymerase II, affinity-purified TFIIH[23] and highly purified CRSP alone (lanes 1 and 2) or with CRSP in the presence of recombinant TBP (lanes 3 and 4) or in the presence of immunopurified TFIID (lanes 5–7).

subcomplex during glycerol gradient sedimentation and gel filtration (data not shown). Most important, immunodepletion of a highly purified CRSP fraction with anti-CRSP150 antibody markedly reduced cofactor activity (Fig. 4b, c). This result indicates that the complex co-precipitating with CRSP150 contributes to the transcriptional cofactor function of CRSP. We cannot exclude the possibility that other weakly associated polypeptides that are not found stably associated with CRSP150 may also contribute to CRSP activity.

So far, our identification and purification of CRSP relied largely on a reconstituted transcription system consisting of partially purified basal factors. As a step towards developing a fully defined transcription system to study activators and their co-activator requirements, we tested the activity of CRSP in a highly purified system. This consisted of recombinant TFIIA, B, E and F subunits, Sp1, affinity-purified TFIIH[23], conventionally purified RNA polymerase II, and highly purified CRSP, in the presence of recombinant TBP or immunopurified TFIID (Fig. 4d). Our results indicate that both CRSP and TFIID are required to mediate Sp1-dependent activation in this highly purified transcription system, thus establishing CRSP as an essential cofactor for Sp1-mediated transcription.

The subunit composition of CRSP indicates that distinct cofactor complexes contain common subunits. For example, CRSP contains three subunits that are also present in yeast or the putative murine mediator complex[7,8,18], CRSPs 33, 77 and 150, but not other mediator subunits such as Srb proteins, Med proteins and Gal11 (ref. 16). Yeast and murine mediator, on the other hand, do not appear to contain homologues of CRSP70, CRSP130 and the p200 subunit of CRSP. The CRSP/mediator comparison is reminiscent of the recently uncovered relationship between TFIID and the SAGA/PCAF complex required for transcriptional stimulation: some of the TAF_{II} subunits of TFIID are also integral components of the SAGA/PCAF complex[24]. In addition, CRSP shares subunits with NAT[19] (CRSP150) and the DRIP/TRAP co-activator[21,22] (p200 subunit of CRSP). As the sequences for many of the NAT-, TRAP- and DRIP-complex subunits have not yet been published, it is possible that there may be other shared subunits. However, both TRAP and DRIP are larger complexes containing a number of subunits with molecular masses that are not found in CRSP.

An increasing number of molecules with co-activator functions, such as the TAF_{II}s, CBP, SRC, PC4, SRBs and TRAP/DRIP subunits, have been identified[5,14,21,25–27]. It was, however, unclear which mechanisms might have evolved to generate the diversity and specificity of co-activators necessary to accommodate the large number of gene-specific promoters and their attendant activators in animal cells. Our results indicate that one way in which co-activator complexes with distinct activities could be assembled could be the mixing and matching of co-activator subunits or subcomplexes in a combinatorial fashion. The finding that CRSP, a co-activator complex required for Sp1 activation, contains several potentially unique subunits, as well as some subunits in common with the yeast and mouse mediators, DRIP/TRAP complexes and the NAT complex, supports the principle that distinct composite co-activator complexes may be assembled by a combinatorial mechanism. As part of such a model, we envisage that different co-activator complexes are directed to assemble at specific promoters by virtue of their interactions with activators, with each other, with core promoter sequences and with the basal machinery. An alternative model would invoke a large collection of partially or completely pre-assembled cofactors. It will be important to determine how CRSP functions in other contexts. Does it function with other activators and, if so, how do different activators use the same cofactor? □

Methods

***In vitro* transcription and preparation of transcription factors.** *In vitro* transcription assays were done with a HeLa fractionated system as described[28], with few modifications. The P1M fraction was prepared by loading a nuclear extract on a P11 (Whatman) column at 0.3 M KCl concentration, washing the extract with 0.5 M KCl and eluting it at 1 M KCl. For the RNA polymerase II basal factor fractions, the extract was dialysed to 20 mM KCl, applied to a P11 column at 0.1 M KCl, washed at 0.3 M KCl and eluted at 0.5 M KCl. Sp1 was overexpressed in HeLa cells using a vaccinia-virus-expressing vector and purified to near homogeneity as described[4]. Immunopurified TFIID was prepared from the P1M fraction using a monoclonal antibody against $hTAF_{II}130$ immobilized on protein A–Sepharose resin (Pharmacia) and eluted with a 20-mer peptide containing the epitope. dTFIIA was overexpressed in *Escherichia coli* and prepared as described[29]. Transcription assays were performed as described[2]. The DNA templates used included $(GC)_3$ BCAT[3], which contains three Sp1-binding sites upstream of the E1B TATA box, and +15 BCAT, which lacks Sp1-binding sites and contains a 15-base-pair insertion producing a longer transcript. Typical reactions (25 μl) contained 50 ng of each template, 5 ng dTFIIA, 50 ng Sp1, 300 ng RNA polymerase II basal factor fraction, and 5–25 ng immunopurified TFIID, or 10 ng recombinant TBP plus varying amounts of CRSP fractions. For the experiment shown in Fig. 1c, 5–200 ng of recombinant PC4 was used[13]. For transcription assays in the purified system, recombinant hTFIIB, F and E and RNA polymerase II fractions were prepared as described[30]. TFIIH was affinity-purified as described[23]. Reactions contained 50 ng dTFIIA, 50 ng Sp1, 5 ng TFIIB, 50 ng TFIIE34, 50 ng TFIIE56, 80 ng TFIIF, 1 ng immunopurified TFIIH, 5 ng immunopurified TFIID, 5 ng RNA polymerase II and 50 ng of a highly purified MonoS CRSP fraction. The reaction products were detected by primer extension. Each transcription reaction was repeated several times, and representative data are shown.

Purification of CRSP. The P1M fraction was used as starting material and HEMG (25 mM HEPES, 12.5 mM $MgCl_2$, 0.2 mM EDTA and 10% glycerol, pH 7.6) supplemented with KCl was used for all of the purification steps, except during Ni-NTA-agarose purification, when EDTA was omitted. The CRSP-containing P1M fraction was loaded on a Ni-NTA-agarose resin (Qiagen) in the presence of 5 mM imidazole, 0.1% N-P40 and 5 mM β-mercaptoethanol, washed with 25 mM HEPES, 12.5 mM $MgCl_2$, 0.7 M KCl and 10 mM imidazole, and eluted with the same buffer containing 100 mM imidazole and 0.15 mM KCl. The Ni-NTA fractions containing CRSP activity were loaded directly onto a Cibacron Blue Sepharose column (Pharmacia), washed with HEMG/0.2 M KCl and eluted with HEMG/0.5 M KCl. The protein peaks were pooled, dialysed to HEMG/0.2 M KCl, applied to Poros heparin (Perceptive Biosystems) and eluted with a gradient from 0.2 M to 1 M KCl. Heparin fractions containing CRSP activity (0.5 M) were pooled and applied to a 15–30% glycerol gradient (in HEMG/0.2 M KCl) and centrifuged at 50,000 r.p.m. for 7 h (TLS55 rotor, Beckman). The active fractions were pooled and applied to MonoS PC 1.6/5 (Pharmacia) and eluted with a gradient from 0.2 M to 1 M KCl. Further details of the purification will be published elsewhere.

Cloning of cDNAs encoding CRSP subunits. MonoS fractions obtained from 400 litres of HeLa cells were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and stained with Ponceau S (Sigma). The bands corresponding to the CRSP subunits were excised and digested with LysC protease. Peptides eluted from the membrane were resolved by reversed-phase HPLC and microsequenced. The amino-acid sequences of peptides of the identified seven CRSP subunits (see Supplementary Information) were used to determine corresponding EST sequences using the BLAST program[7,21,22]. A partial clone of *CRSP150* was identical to the *EXLM1* gene (GenBank accession number, AB006651). Full-length *CRSP34* was assembled from EST clones 147,371 and 182,652. *CRSP77* was assembled using human EST clone 262,073 and those clones represented in the THC 192416 alignment (Institute for Genome Research). For *CRSP130* and *CRSP70* sequences, cDNAs obtained from EST clones 767,814 and 332,225, respectively, were used to probe a human testis library (Clontech). Positive clones were cloned into the pBluescript KS^+ plasmid (Stratagene), sequenced and aligned.

Antibody generation and immunoprecipitation. Residues 1,306–1,454 of CRSp150, residues 366–500 of partial CRSP130, and residues 51–167 of CRSP33 were overexpressed as either His_6 tag (Invitrogen) or glutathione-*S*-transferase (GST; Pharmacia) fusion proteins in *E. coli*. Rabbits were boosted five to seven times with affinity-purified antigen. The antisera obtained were affinity-purified using antigens immobilized on Affigel 10 or 11 resin (Biorad). For the immunoprecipitation assay shown in Fig. 1a, 100–200 ng affinity-purified

anti-CRSP150 antibody was incubated with 100 μg of CRSP fractions derived from the Ni-NTA-agarose chromatography. The immunoprecipitated complex was recovered by addition of protein A–Sepharose, washed extensively with HEMG/0.7 M KCl or HEMG/1 M KCl and extracted with 100 mM glycine buffer, pH 2.5. For the immunodepletion studies shown in Fig. 4b, c, the CRSP fraction obtained after Cibacron Blue Sepharose chromatography was concentrated over a MonoS column. 200 ng of eluted material was incubated with 100 ng affinity-purified anti-CRSP150 antibody or control antibody (unrelated rabbit serum IgG). Antibody–antigen complexes were recovered using protein A–Sepharose and the supernatant was assayed for CRSP activity in transcription assays.

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