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Identification and characterization of RED120: A conserved PWI domain protein with links to splicing and 3′-end formation

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Abstract **Precursor (pre)-mRNA splicing can impact the efficiency of coupled steps in gene expression. SRm160 (SR-related nuclear matrix protein of 160 kDa), is a splicing coactivator that also functions as a 3′-end cleavage-stimulatory factor. Here, we have identified an evolutionary-conserved SRm160-interacting protein, referred to as hRED120 (for human Arg/Glu/Asp-rich protein of 120 kDa). hRED120 contains a conventional RNA recognition motif and, like SRm160, a PWI nucleic acid binding domain, suggesting that it has the potential to bridge different RNP complexes. Also, similar to SRm160, hRED120 associates with snRNP components, and remains associated with mRNA after splicing. Simultaneous suppression in *Caenorhabditis elegans* of the ortholog of hRED120 with the orthologs of splicing and 3′-end processing factors results in aberrant growth or developmental defects. These results suggest that RED120 may function to couple splicing with mRNA 3′-end formation.**

Keywords: Splicing; 3′-End processing; RNAi; snRNPs; SRm160

1. Introduction

The processing of precursor (pre-) mRNA to mature mRNA involves a series of highly integrated and coupled steps, including the addition of a 5′-m7G cap, intron removal by splicing, and 3′-end cleavage and polyadenylation. Most pre-mRNAs in higher eukaryotes contain at least one intron that must be excised by a spliceosome. The major spliceosome consists of the five small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5, as well a large number of non-snRNP protein splicing factors (for reviews see [1–3]). Members of the SR (Serine/Arginine) family of splicing factors, as well as SR-related proteins, are among the best characterized non-snRNP splicing factors and have well defined roles in splice site selection, and in the coupling of splicing to other steps in gene expression (reviewed by [4–7]).

Splicing influences coupled steps in gene expression by several mechanisms. For example, several splicing factors have been described that affect 3′-end processing [8,9]. The 3′-end processing machinery is relatively well-conserved from yeast to higher eukaryotes, and is composed of five different factors: Poly(A) polymerase, cleavage and polyadenylation specific factor (CPSF), cleavage stimulation factor (CstF) and cleavage factors I and II (CFI and CFII). These factors associate with the bipartite poly (A) signal in the nascent transcript and catalyze the coupled 3′-end cleavage and polyadenylation reaction (reviewed by [10,11]). Direct interactions between splicing factors and 3′-end processing factors may result in the stimulation of cleavage or polyadenylation. These interactions include the binding of the U2 snRNP auxiliary factor 65 kDa subunit to poly(A) polymerase and CFI and interactions between U2 snRNP and CPSF [12–14].

The carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) and the mRNA binding exon-junction complex (EJC) also mediate effects of splicing on coupled steps in gene expression [8,15]. The EJC is composed of a set of factors that associate with pre-mRNA during or soon after transcription and which remain bound to mRNA after splicing [16]. The EJC is deposited 20–24 nucleotides upstream of spliced exon junctions [17–19], and is known to contain factors implicated in mRNA export, 3′-end formation, mRNA turnover via nonsense-mediated decay (NMD), and translation [20–24].

SRm160 (the SR-related nuclear matrix protein of 160 kDa) is a splicing coactivator that associates with snRNP components, assembled splicing complexes, and the EJC [18,19,25–28]. In addition to its role in the splicing of specific pre-mRNAs, SRm160 promotes 3′-end cleavage via its conserved N-terminal PWI domain [28]. In order to understand how SRm160 functions, we have recently used mass spectrometry to identify factors that associate with this protein [49]. This resulted in the identification of hRED120 (human Arg/Glu/Asp-rich protein of 120 kDa), which shares several similarities with SRm160. In addition to interacting with SRm160, hRED120 associates with snRNPs in the absence of pre-mRNA, with assembled splicing complexes containing the pre-mRNA

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substrates, intermediates and products of in vitro splicing reactions, and remains preferentially bound to spliced mRNA. We further show that simultaneous repression by RNA interference (RNAi) in the nematode *Caenorhabditis elegans* of orthologs of hRED120 and the SR family splicing factor SRp20, or of the orthologs of hRED120 and the 3′-end processing factors CstF50 and Clp1, results in growth impairment or embryonic lethality. Together, our results provide evidence for the importance of interactions between RED120 and factors involved in pre-mRNA processing and further suggest a possible role for RED120 at the interface of splicing and 3′-end formation.

2. Materials and methods

2.1. Cell extracts and antibodies

HeLa nuclear extract was purchased from C4 (Belgium). The following antibodies were used in this study: αSRm160 murine monoclonal (mAb-B1C8; [29,30]), αSRm160 rabbit polyclonal (αSRM160; [25]), αSRm300 rabbit polyclonal (αSRm300; [31]), rabbit polyclonal α116 kDa-U5 snRNP-specific protein [32], rabbit polyclonal αCBP80 [33], polyclonal αRNPS1 [34], polyclonal αUAP56 [35], monoclonal αY14 (mAb-4C4 from G. Dreyfuss), monoclonal αSm proteins (Y12; [36]) and monoclonal αSC35 [37]. Antibodies specific for hRED120 were raised by immunization of two New Zealand white rabbits with 200 μg of nhRED120 or chRED120 proteins (see below) per immunization. Five immunizations were administered.

2.2. Database searches

Putative RED120 family members were identified using Blast searches [38]. Proline-rich regions, RNA recognition motifs and PWI domains [39] were identified using the Prosite database [40]. Low complexity regions were identified using the SEG program [41]. Multiple sequence alignment and a phylogenetic tree were calculated using ClustalX [42].

2.3. DNA constructs

RNA isolated from HeLa cells (ATCC) was reverse transcribed with an oligonucleotide complementary to the 3′UTR of hRED120 and hRED120 cDNA was amplified by PCR using oligonucleotides (Sigma) ''5′hRED120'' and ''3′hRED120''. 5′hRED120 contained a SpeI recognition site and hRED120 translation initiation sequences. 3′hRED120 contained a KpnI recognition site and sequences complementary to the hRED120 translation termination region. The resulting PCR product was digested with SpeI and KpnI (New England Biolabs) and cloned into the multiple cloning site of pBluescript IIKS (Stratagene). Positive pBShRED120 clones were verified by sequencing. hRED120 5′ end sequences were isolated from pBShRED120 after digestion with SpeI and HindIII. The resulting fragment was subcloned into the PstI and HindIII sites of pQE30 (Qiagen) to produce pQEnhRED120. SauIIIA–KpnI digestion of pBShRED120 served to isolate hRED120 3′ end sequences which were then subcloned into pQE31 (Qiagen) to generate pQEchRED120.

2.4. Protein expression and antibody production

E. coli strain BL21/pREP4 (Qiagen) was transformed with pQEnhRED120 and pQEchRED120 and grown in LB medium supplemented with ampicillin at 37 °C to an OD_{600} of 0.06 and at room temperature to an OD_{600} of 0.8. Protein expression was induced with 0.5 mM IPTG (Sigma) for 3 h. Cell pellets were sonicated in lysis buffer (500 mM NaCl, 5 mM magnesium acetate, 1 mM PMSF and 50 mM Tris–HCl, pH 7.5) and cell lysates were recovered after 20 min of centrifugation at 9000 rpms with a SS34 rotor. Extracts were adjusted to 20 mM imidazole (Sigma) and mixed with 200 μl of buffer-washed packed Nickel-NTA beads (Invitrogen) per liter of bacterial culture. The mixture was incubated for 90 min at 4 °C and the beads were recovered in an Econo-Column (Biorad). The beads were washed twice in 10 vol. of PBS-20% Glycerol (PBS-G) and protein was eluted in the same buffer supplemented with 0.4 M imidazole and dialyzed against PBS-G.

2.5. Protein detection and immunolocalization

hRED120 was detected by Western blotting using rabbit αnhRED120 or αchRED120, diluted 1:1000. Detection was performed using a goat αrabbit secondary antibody coupled to peroxidase, diluted 1:2000 (Sigma). Western blots with antibodies to SRm160, RNPS1, UAP56, Y14, U5-116 kDa protein, CBP80, and Sm proteins were performed as described previously [25,32–36].

Immunofluorescence localization was performed as described previously [43] with pre-immune serum or αchRED120 diluted 1:250 and αSC35 diluted 1:3. Secondary antibodies were α mouse and α rabbit coupled to Texas red or FITC, respectively, diluted 1:100 (Sigma). Preparations were analyzed using a Zeiss LSM 510 confocal microscope.

2.6. Immunoprecipitations

Interactions between hRED120, SRm160, SRm300, RNPS1, Y14, and UAP56 were analyzed as follows: 30 μl of packed protein A Sepharose beads were loaded with a mixture of αchRED120 and αnhRED120 sera (14 μl), antigen affinity-purified rabbit polyclonal αSRm160 (50 μg), αSRm300 serum (25 μl), and, as a control for non-specific immunoprecipitation, with rabbit αmouse IgG and IgM (72 μg). Antisera were cross-linked to protein A beads with 20 mM dimethylpimelidate (Harlow and Lane). Rabbit αmouse IgG and IgM (150 μg) was coupled to protein A-Sepharose prior to coupling of B1C8, which is an IgM monoclonal. Beads were mixed with 1.5 mg of nuclear extract preincubated for 15 min at 30 °C under splicing conditions (2 mM $MgCl_2$, 1.5 mM ATP, 5 mM phosphocreatine, 16 ng/μl RNase cocktail (Boehringer), DNase I (0.3 U/μl), 1 mM potassium fluoride, 0.1 mM sodium pyrophosphate, and 1 mM sodium β-glycerophosphate). The extract was incubated with the beads for 3 h at 4 °C with gentle rotation in 60 mM NaCl, 13 mM HEPES, pH 7.9, 1.4 mM $MgCl_2$, 14% glycerol, 0.5 mM DTT, 0.7 mM β-glycerol phosphate, 0.7 mM NaF, 0.07 mM Na^+ pyrophosphate in a final volume of 750 μl. The beads were washed three times with 1.5 ml of IPWB100 (100 mM NaCl, 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 0.1% NP40) and eluted with 2 M NaCl, 10 mM HEPES, pH 7.5, 1 mM EDTA (200 μl). These pooled eluates were back-bound with Protein A Sepharose or Protein A Sepharose coated with rabbit αmouse IgG and IgM for 30 min at 4 °C with rotation. After elution, the samples were precipitated with trichloroacetic acid (TCA) (20%) and sodium deoxycholate (1.5 mg/ml), washed with 10% TCA, and with acetone, resuspended in SDS sample buffer and analyzed by SDS–PAGE. To co-immunoprecipitate other splicing factors, 8 mg of HeLa nuclear extract was incubated overnight at 4 °C with 400 U of HPRI, 22 μg of *E. coli* tRNA, 100 μg of heparin and 4 mg of preimmune or αchRED120-coupled protein A beads. Beads were recovered in an econo-column and washed three times with 1 ml of buffer D/0.1% NP40 [44]. The beads were eluted with 150 μl of buffer D/0.1% NP40 mixed with NaCl as indicated, or with 150 μl of Glycine 0.1 M, pH 3. Before elution, beads were incubated 15 min at 4 °C. After each elution beads were washed three times with 150 μl of elution buffer. Two percent of the input and one-third of the eluates were analyzed by SDS–PAGE and immunoblotting. Two percent of the input and two-third of the eluates were analyzed by Northern blot (see below). When immunoprecipitations were performed with RNase-treated extract, 6.5 mg of HeLa extracts were incubated for 1 h in a splicing reaction (see below) in the presence of buffer alone, 200 μg of RNase A (Boehringer), or 36 U of RNaseH (Gibco) mixed with 11.4 nmol of 2b oligo (5′CAGATACTACACTTG3′).

To immunoprecipitate from splicing reactions, 200 μg of protein A Sepharose beads (Amersham) were bound to 20 μl of antibody for 1 h at room temperature in buffer D. Then, beads were washed extensively with buffer D and buffer D diluted 1:3 (buffer D/3) and mixed with 20 μl splicing reactions incubated for 1 h (see below). 10 μg of competitor *E. coli* tRNA (Boehringer), 1 U/μl of HPRI (Amersham) and 50 μg/ml of heparin (Sigma) were also added. 50000 cpm of labeled U3 snoRNA were included where indicated (see below). The mix was incubated for 2 h at 4 °C with rotation in a final volume of 200 μl of buffer D/3. After incubation, beads were washed 3 times with 10 vol. of buffer D/3 and twice with 10 vol. of buffer D-200 mM KCl-0.1% NP40. RNA was analyzed from the beads after proteinase K treatment as described [45]. Quantifications were done in a Cyclone Phosphorimager (Perkin Elmer). Percentage of binding was calculated after subtracting the preimmune background from the signal obtained in the RED120 antibody immunoprecipitation.

2.7. Splicing reactions and Northern blots

Splicing reactions were performed as previously described [33]. Pre-mRNAs used were transcribed as previously described [33]. Prior to transcription, pAd2 was digested with SauIIIA, and pMXSVL with BamHI. pMXSVL was transcribed by T7 and pAd2, with T3 (Promega). Northern blots using snRNA-specific riboprobes were performed as previously described [46].

2.8. RNA interference in C. elegans

Generation of templates for gene-specific dsRNAs and RNAi in *C. elegans* were performed as previously described [47,48]. The templates were amplified with the following T3 and T7 promoter containing primers: CeRED120F: caatgcgaaaaatgcgagc; CeRED120R: gtaattccgagacgacggg.

3. Results

3.1. Identification of hRED120 in affinity-purified SRm160-containing complexes

An immunoaffinity purification procedure for gel-free tandem mass spectrometry-based analysis of endogenous complexes was used to identify proteins associated with SRm160 and SRm300 (the serine/arginine-repeat related nuclear matrix proteins of 160 kDa and 300 kDa) splicing coactivators [49]. This resulted in the identification of two polypeptides (underlined in Fig. 1A) corresponding to hRED120. Sequenced transcripts corresponding to hRED120 were previously mapped to a gene (designated s164) within an Alzheimer's-susceptibility locus on chromosome 14 [50]. The full length ORF of hRED120 predicts a protein of 120 kDa that includes a region rich in RE/RD dipeptides in the central part of the protein, a proline-rich region and RNA recognition motif (RRM) at the amino (N-) terminal end, and a PWI domain at the carboxyl (C-) terminal end (Fig. 1A). The PWI motif [39], which is also present in SRm160 and the U4/U6 snRNP-associated PRP3 splicing factor, has been shown previously to function in nucleic acid binding and to facilitate SRm160-dependent stimulation of 3′-end processing [28].

Database searches revealed that hRED120 belongs to a family of RNA binding proteins with members found in yeast to human (Fig. 1B). All of the family members share the RE/RD-rich central region and the C-terminal PWI motif or a PWI-related motif. Although the PWI domain is quite well conserved in most of the metazoan RED120 family members, it is only found by Prosite with medium to high probability in fungal species (Fig. 1C and D). Similarly, the N-terminal RRM found in several of the metazoan RED120 family members is not found in the candidate RED120 orthologs of *Saccharomyces cerevisiae* and Neurospora, although it is present in a candidate *S. pombe* ortholog (Fig. 1B). A potential *S. cerevisiae* ortholog of hRED120 and other PWI motif proteins, Snu71p, has been identified as a U1 snRNP-specific protein [45,51,52]. This fact, together with the potential association of hRED120 with SRm160-containing complexes, as well as the sequence features of the different RED120 family members that resemble other pre-mRNA processing factors, strongly suggest that RED120 functions in pre-mRNA processing.

3.2. hRED120 associates with SRm160/300 and localizes to nuclear speckles

In order to biochemically characterize hRED120, two different rabbit polyclonal antisera were raised against bacterially-expressed 6×His-tagged recombinant fragments of the protein, one containing N-terminal amino acids 1–96 (nhRED120) and the other containing C-terminal amino acids 655–843 (chRED120) (boxed in Fig. 1A). Both antisera, but not the corresponding pre-immune sera, recognized a single band of ~120 kDa in immunoblots of HeLa nuclear extract (Fig. 2A).

To verify whether SRm160 and its partner protein SRm300 associate with hRED120, immunoprecipitates collected with the αhRED120 antibodies from nuclease pre-treated HeLa nuclear extract were immunoblotted with a monoclonal antibody specific for SRm160 (mAb-B1C8) (Fig. 2B, lanes 1–4). A subpopulation of SRm160 in the extract was co-immunoprecipitated with the αhRED120 antibodies. Similarly, immunoprecipitates collected from HeLa nuclear extract with the αSRm160 antibodies rAb-αSRm160 and mAb-B1C8, as well as with a polyclonal antiserum specific for SRm300, contained a subpopulation of hRED120, as detected by immunoblotting with αchRED120 (Fig. 2B, lanes 5–9). Co-immunoprecipitation of hRED120 with the αSRm160/300 antibodies, and of SRm160 with the αchRED120 serum, appeared specific, since excess levels of control sera did not result in co-immunoprecipitation (compare lanes 3 and 4 or lanes 6 and 7–9). Moreover, these interactions are probably not the consequence of non-specific "tethering" by nucleic acid, since the nuclear extracts were pre-treated extensively with RNase and DNase prior to immunoprecipitation. These results indicate that subpopulations of hRED120 and SRm160 proteins specifically associate with each other in HeLa nuclear extract.

A feature of many pre-mRNA processing factors, including SRm160 and SRm300, is their localization to interchromatin granule clusters or "speckles". To determine whether hRED120 also possesses this property, we immunolabeled HeLa cells with αchRED120 antisera, using a monoclonal antibody to the SR family splicing factor SC35 as a marker for speckles. These immunostainings reveal that hRED120 is concentrated in nuclear speckles, and further suggest a role for hRED120 in pre-mRNA processing in vivo (Fig. 2C). In addition to the prominent speckle staining pattern, a faint αchRED120 immunostaining signal is detected in the cytoplasm, above the background signal obtained with the pre-immune serum. This suggests that hRED120 could also have a role in the cytoplasm (Fig. 2C and data not shown).

3.3. Association of hRED120 with multiple splicing components

In order to determine whether hRED120, like SRm160/300, can associate with functional splicing complexes, the ability of the αchRED120 antibody to immunoprecipitate splicing complexes formed in HeLa nuclear extract was next tested. Radiolabeled Ad2 pre-mRNA, as well as an equivalent amount of U3 snoRNA as a non-specific control, was incubated in splicing reactions for 1 h and the mixture was added to protein A-Sepharose beads pre-coated with αchRED120 or control antibody. The αchRED120 antibody immunoprecipitates splicing intermediates with similar efficiency as a positive control antibody (Y12), which is specific for the snRNP Sm proteins (Fig. 3A, compare lanes 3 and 4). Similar results were obtained with the αnhRED120 antibody, although this reagent did not immunoprecipitate complexes containing splicing intermediates as efficiently as αchRED120 (data not shown). Relatively little pre-mRNA and splicing intermediates or products were immunoprecipitated with the pre-immune serum and none of the antisera tested immunoprecipitated significant levels of the U3 snoRNA (Fig. 3A). These results indicate that

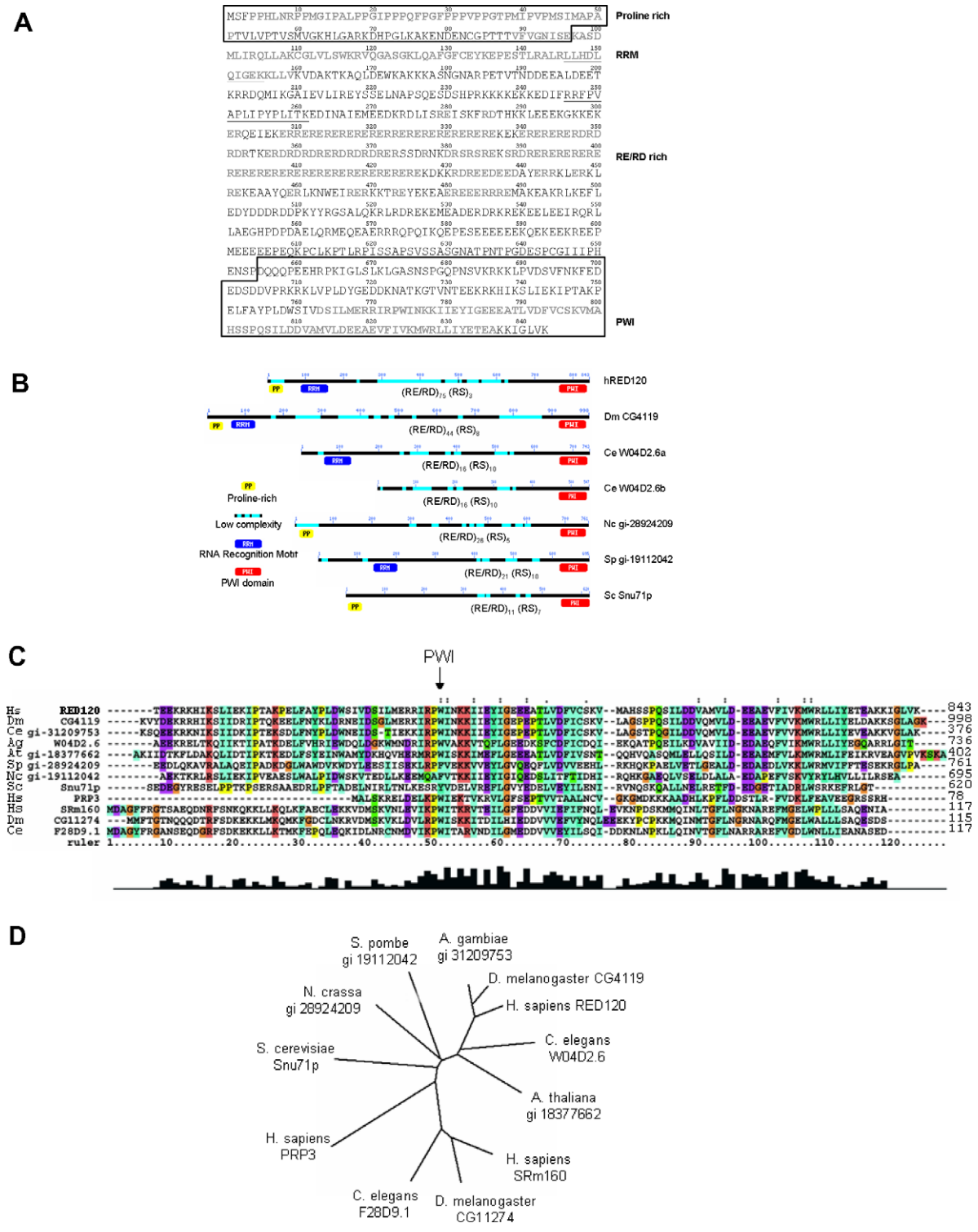

Fig. 1. The RED120 family of proteins. (A) Amino acid sequence and protein domains of human RED120 (hRED120). Proline rich region, RNA recognition motif (RRM), RE/RD dipeptides and PWI domain are highlighted in bold. Peptides identified by mass spectrometry are underlined. Amino- and carboxy-terminal regions used to raise antibodies are boxed. (B) Schematic representation of known full-length RED120 proteins from human (hRED120), fruit fly (Dm CG4119), *C. elegans* (two predicted variants from open reading frame W04D2.6), the fungus *Neurospora crassa* (Nc, gi number 28924209) and the yeasts *S. pombe* (Sp, gi number 19112042) and *S. cerevisiae* (Sc Snu71p). Proline-rich regions (PP), RNA recognition motifs (RRM), PWI domains and low complexity regions are indicated for each homolog. Occurrences of RE/ER, RD/DR or RS/SR dipeptides present in the entire proteins are indicated under each protein bar. (C) Multiple sequence alignment of PWI-like domains. Clustal X comparison of PWI-like motifs present in the C-terminal regions of the RED120 proteins from Fig. 1B supplemented with those of the mosquito *Anopheles gambiae* (Ag gi number 31209753) and the plant *Arabidopsis thaliana* (At gi number 18377662). In addition, PWI domains from the N-termini of human splicing factor PRP3 and SRm160 are co-aligned, together with *C. elegans* and *D. melanogaster* homologs of the latter (Ce F28D9.1 and Dm CG11274). (D) Dendrogram of PWI-like sequences from the multiple sequence alignment shown in panel C.

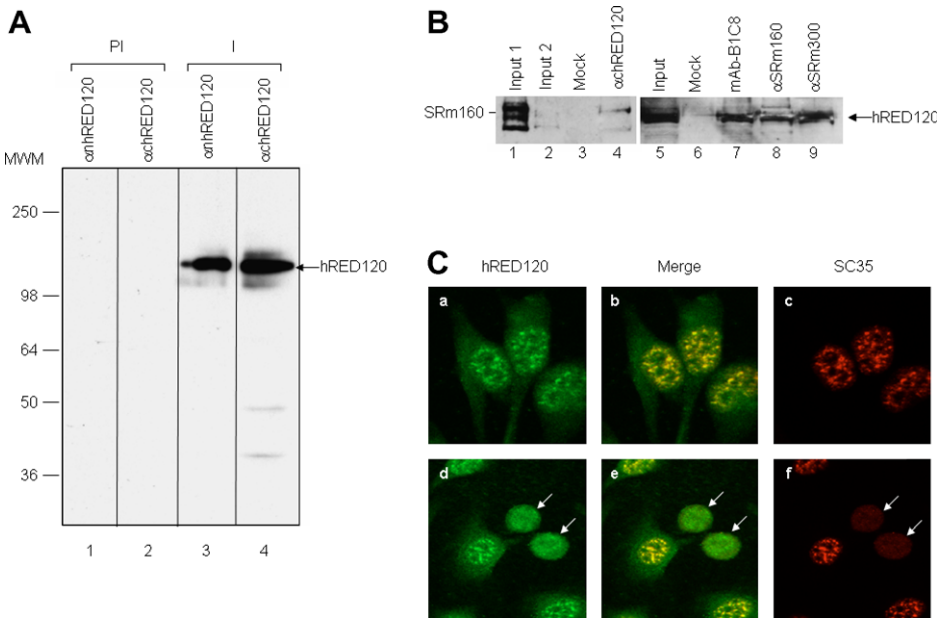

Fig. 2. hRED120 associates with the splicing coactivator subunits SRm160 and SRm300, and localizes to nuclear speckles. (A) hRED120 antibodies recognize a single band of 120 kDa. Immunoblotting of HeLa nuclear extract with preimmune rabbit serum (PI) or rabbit serum after immunization (I) with the N-terminal (αnhRED120) or C-terminal (αchRED120) regions of hRED120. The position of hRED120 is indicated to the right. The sizes of molecular weight markers (MWM) are indicted to the left. (B) hRED120 coimmunoprecipitates with SRm160 and SRm300. HeLa nuclear extract was immunoprecipitated with αchRED120 (lane 4), a monoclonal antibody αSRm160 (mAb-B1C8, lane 7), a polyclonal antibody αSRm160 (lane 8), or αSRm300 (lane 9), or with an excess of a control antibody (lanes 3 and 6). The immunoprecipitates were immunoblotted with antibodies specific for SRm160 (lanes 1–4) or hRED120 (lanes 5–9). Inputs correspond to 5% (input 1 in lane 1), or 1% (input 2 in lane 2 and input in lane 5) of the nuclear extract used for each immunoprecipitation. The position of SRm160 and hRED120 is indicated to the side of the figure. (C) hRED120 localizes to speckles. HeLa cells were fixed and stained with antibodies against hRED120 (green stain in a and d) or SC35 (red stain in c and f). Overlapping images are also shown (merge in b and e). Arrows point two cells after cytokinesis (d–f) with a homogeneous nuclear staining for both hRED120 and SC35.

hRED120 specifically associates with functional splicing complexes.

Given our previous observation of a synthetic lethal interaction between Snu71p, the candidate ortholog of hRED120 in yeast, and yeast nuclear cap binding complex (yCBC) [53], we speculated that these components could form a conserved physical interaction in mammals. Similarly, since Snu71p in yeast is a component of U1 snRNP, and SRm160 associates with snRNP components in mammalian extracts, it is likely that hRED120 also associates with one or more snRNP components in mammalian extracts. Thus, we determined whether splicing factors such as the CBC 80 kDa subunit (CBP80) or snRNPs associate with hRED120, and we also assessed the relative stabilities of these interactions. Immunoblotting of HeLa nuclear extract proteins eluted from protein A–Sepharose beads bound to αchRED120 indicated that most of the immunoprecipitated hRED120 in the nuclear extract remains bound to the beads at 2.0 M salt; only treatment with 0.1 M glycine pH 3.0 resulted in removal of most of the protein (Fig. 3B, upper panel). In contrast, immunoblotting of the different salt eluates indicated that CBP80 forms a salt-sensitive association with hRED120 (most of the protein is eluted at 0.35–1 M salt; Fig. 3B, second panel). Similarly, immunoblotting the eluates with the αSm Y12 antibody revealed that Sm proteins are also bound to hRED120Fig. 3B, and that most of these proteins co-elute with CBP80 at 0.35–1 M salt (third panel). To assess whether the binding of Sm proteins to hRED120 represents a specific interaction between hRED120 and one or more snRNPs, RNA recovered from the αchRED120 eluates was analyzed by Northern blotting with a mixture of ^{32}P-labeled riboprobes specific for U1, U2, U4, U5 and U6 snRNAs (Fig. 3B, lower panel). Although some non-specific immunoprecipitation of U5 and U6 snRNAs was observed, hRED120 appears to associate with all five snRNAs in the extract with a similar salt sensitivity as Sm proteins and CBP80. This suggests a possible interaction of hRED120 with one or more general snRNP components, or else that the co-immunoprecipitated snRNAs reflect an association between hRED120 and pre-assembled splicing complexes containing all five snRNPs.

Since hRED120 contains RRM and PWI motifs, both of which can bind RNA, we also addressed whether the association of hRED120 with snRNP components is RNA-mediated. HeLa nuclear extracts were pre-incubated with RNase A, or with RNase H in the presence of an oligonucleotide complementary to U2 snRNA (nucleotides 28–42; U2b), which pairs with the branch region in pre-mRNA. Thus, RNase H-mediated cleavage of U2 snRNA results in an extract that is deficient in splicing (data not shown). Next, immunoprecipitation was carried out with αchRED120 antibodies. Immunoblotting of the immunoprecipitates with antibodies against the U5 snRNP-specific 116 kDa protein [32] and Sm proteins indicated that the RNase A or RNase H+U2b oligonucleotide pre-treatments did not result in a reduction in the association between hRED120 and these snRNP proteins (Fig. 3C). However, RNase A treatment abolished the interaction between hRED120 and CBP80 (Fig. 3C). It therefore appears that the association between hRED120 and CBP80 requires

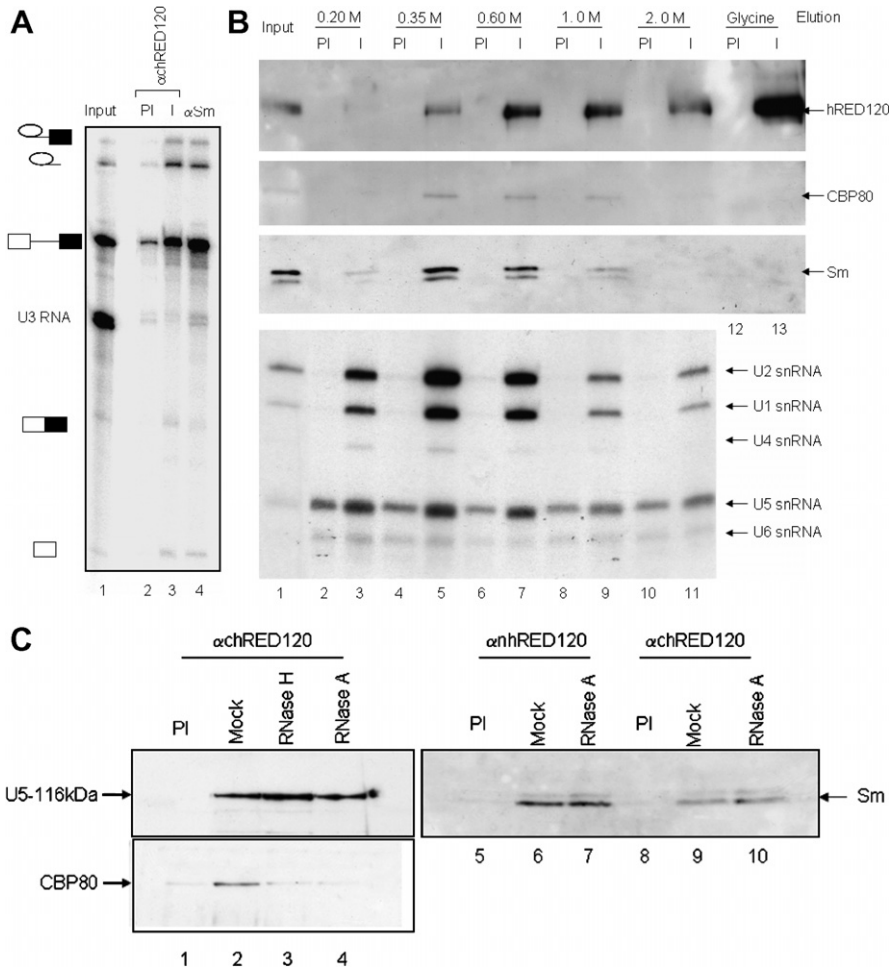

Fig. 3. Association of hRED120 with snRNPs and assembled splicing complexes. (A) hRED120 associates with splicing complexes containing pre-mRNA, intermediates and products of the splicing reaction. Radiolabeled adenovirus splicing substrate mixed with U3 snoRNA was incubated with nuclear extracts. After the splicing reaction, the extracts were immunoprecipitated with preimmune (PI, lane 2) or immune αchRED120 serum (I, lane 3) or αSm antibody as positive control (lane 4). Immunoprecipitates were separated by electrophoresis and labeled RNAs were visualized. 30% of the input was also loaded (lane 1). Position of splicing substrate, products and intermediates is indicated to the left. (B) hRED120 coimmunoprecipitates with several components of the splicing machinery. Nuclear extracts were bound to a preimmune (PI) or an αchRED120 antibody (I) column and eluted with increasing salt concentrations or Glycine as indicated at the top of the figure. Proteins and RNAs were purified from the eluates. Proteins were resolved by SDS–PAGE and hRED120 (upper panel), CBP80 (second panel) or Sm proteins (third panel) were detected by Western blotting. The RNA components were resolved by PAGE and revealed by Northern blotting with a mixture of labeled U1, U2, U4, U5 and U6 snRNAs (lower panel). Two percent of the input was also loaded (lane 1). Position of the proteins and snRNAs detected is indicated to the right. (C) hRED120 co-immunoprecipitates with some splicing components in an RNA-independent manner. Nuclear extracts were incubated with or without RNase A or with RNase H in the presence of an oligonucleotide directed to the branch point binding sequences of U2 snRNA. Then, an immunoprecipitation was carried out with either pre-immune serum (PI, lanes 1, 5 and 8) or antibodies against the N-terminal (αnhRED120, lanes 6 and 7) or the C-terminal (αchRED120, lanes 2–4 and 9 and 10) regions of hRED120. The immunoprecipitates were run on SDS–PAGE, Western blotted and developed with antibodies against the U5-116 kDa specific protein (upper panel, lanes 1–4) CBP80 (lower panel, lanes 1–4), or Sm proteins (lanes 5–10). Position of the detected proteins is indicated to the side of the figure.

RNA, whereas the association between hRED120 and snRNP components may be RNA independent.

3.4. Immunodepletion of hRED120 does not prevent splicing or cleavage of specific pre-mRNAs

Since hRED120 associates with several splicing components and assembles into functional splicing complexes, we hypothesized that it may have a role in pre-mRNA processing. To initially test this, HeLa nuclear extract was efficiently immunodepleted of hRED120 with αchRED120 (Supplementary Fig. 1). However, no differences in the overall efficiencies or rates of splicing of a constitutively spliced (Ad2-derived) or ESE-dependent (Drosophila *doublesex*-derived) pre-mRNA were observed (Supplementary Figs. 2 and 3), whereas SRm160 was shown previously to be important for the splicing of both of these pre-mRNAs [25,26]. Similar results were obtained for other substrates tested, including Ftz and Msl-2 (data not shown). We also used the depleted extracts to evaluate the role of hRED120 in 3′-end cleavage. The efficiency of cleavage of the L3 poly (A) site was similar in mock depleted and hRED120-depleted extracts (data not shown). These results indicate that hRED120 is not part of a common network of interactions involving SRm160 that is required for the stimulation of 3′-end cleavage or for the splicing of specific pre-mRNAs. However, it remains possible that hRED120 is important for the constitutive and/or alternative splicing as

well as for 3′-end processing of pre-mRNAs not tested in the present study.

3.5. Association of RED120 with spliced mRNA

Since SRm160 is an EJC component and associates with hRED120, we next tested whether hRED120 also associates with spliced mRNA. This also seemed possible given that immunoprecipitation with the αchRED120 antibody, compared to the αSm (Y12) antibody, resulted in some enrichment of the ligated exon product of splicing reaction (compare lanes 3 and 4 in Fig. 3A). Spliceosomal snRNPs remain preferentially associated with the excised lariat product of the splicing reaction. Consistent with this, enrichment of the lariat product of the splicing reaction by immunoprecipitation was observed with the αSm antibody (Fig. 3A). To determine whether the association of hRED120 with the exon-product RNA is dependent on prior splicing, the adenovirus-derived MXSVL-38 (containing a 38 nt 5′-exon) pre-mRNA was incubated in a splicing reaction, and a cDNA-derived exon-product of identical sequence was incubated in a parallel reaction. Immunoprecipitations were performed with pre-immune and αchRED120 antibody from each reaction (Fig. 4A). Quantification of the results indicated that hRED120 associates with the exon product RNA generated by splicing more efficiently than the cDNA-derived exon product RNA (Fig. 4B).

The association of RED120 with spliced mRNA suggests that RED120 could be an EJC associated factor. However, RNaseH cleavage-mapping experiments did not reveal a specific interaction between RED120 and a region of the mRNA including the EJC ''docking site'', suggesting that its association with spliced mRNA may not be restricted to this region (data not shown). Nevertheless, RED120 may associate with the EJC since it can interact with EJC proteins besides SRm160. The αchRED120 antibody was used to collect immunoprecipitates from HeLa nuclear extract pre-treated with RNase and DNase (see Section 2). The immunoprecipitates were immunoblotted with anti-sera specific for REF, Y14, RNPS1 and UAP56. Bands consistent with the sizes of all four of these EJC components were detected in the immunoprecipitates (Fig. 4C and data not shown).

3.6. Interactions involving hRED120 and pre-mRNA processing factors in the development of C. elegans

The results described above provide evidence that hRED120 is associated with several factors involved in splicing and/or coupled steps in gene expression, including SRm160, snRNPs and EJC components. It was therefore of interest to determine whether interactions between RED120 and other pre-mRNA processing factors are physiologically relevant in the context of a whole organism. Analysis of the *C. elegans* genome

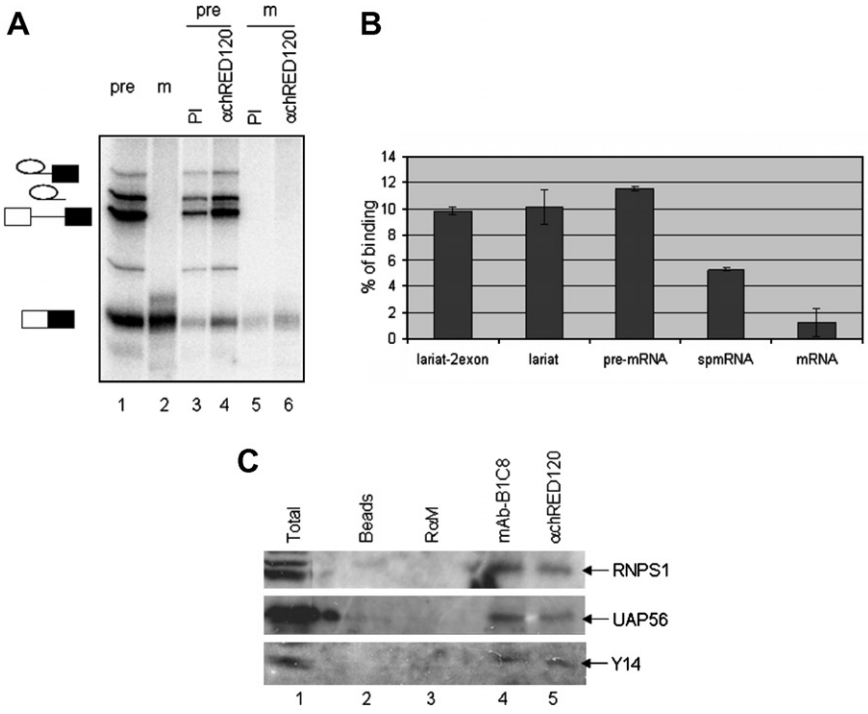

Fig. 4. hRED120 associates with the exon junction complex (EJC). (A) hRED120 binds spliced transcripts more efficiently than unspliced transcripts. Labeled MXSVL-38 splicing substrate or its spliced counterpart were incubated with nuclear extract. After the splicing reaction, the extracts were immunoprecipitated with preimmune serum (PI) or with αchRED120. Labeled RNAs isolated from 1/3 of the input (lanes 1 and 2) or from the immunoprecipitates (lanes 3–6) were visualized. Position of splicing substrates and products is indicated to the left. (B) Quantification of the results shown in panel A. Percentage of binding was calculated for the splicing substrate (pre-mRNA), splicing intermediate (lariat-2exon), splicing products (lariat and spliced mRNA (spmRNA)) and unspliced mRNA (mRNA). The preimmune background has been subtracted from the immunoprecipitated bands before determination of the percentage of binding. Error bars indicate standard deviations of three different experiments. (C) hRED120 coimmunoprecipitates with RNPS1, UAP56 and Y14. HeLa nuclear extract was immunoprecipitated with αchRED120 (lane 5), a monoclonal antibody αSRm160 (mAb-B1C8, lane 4), an excess of a control antibody (RαM, lane 3) or with Protein A–Sepharose without coupled antibody (beads, lane 2). The immunoprecipitates were immunoblotted with antibodies specific for RNPS1 (upper panel), UAP56 (middle panel) or Y14 (lower panel). Total nuclear extract in lane 1 corresponds to 5% of the input used in each immunoprecipitation. The position of RNPS1, UAP56 and Y14 is indicated to the side of the figure.

Table 1
Codepletion of RED120 and pre-mRNA processing factors by RNAi

RNAi	Gene ID	Phenotype
RED120	W04D2.6	Wild-type[a]
RED120 + SRm160	F20D9.1	Wild-type
RED120 + RNPS1	K02F3.11	Wild-type
RED120 + Ref1 + Ref2 + Ref3	C01F6.5, F23B2.6, M18.7	Wild-type
SRp20	C33H5.12	Wild-type
RED120 + SRp20		Slow growth
RED120 + SRp40	W02B12.2	Wild-type
RED120 + SC35	EEED8.7	Wild-type
Clp1	F59A2.4	Wild-type
RED120 + Clp1		100% embryonic lethal
CstF50	F28C6.3	Wild-type
RED120 + CstF50		Aberrant oogenesis Slow growth
RED120 + CFIm68	D1046.1	Wild-type

[a]Reported in wormbase as wt, and also slow growth when RNAi performed on RNAi hypersensitive strain *rrf-3*.

revealed two predicted variants from a single ORF similar to hRED120 (Fig. 1B). The longer of the putative *C. elegans* ortholog sequences, referred to below as CeRED120, displayed high homology (82% similarity) with hRED120. Depletion of CeRED120 by injection of dsRNA into wild-type strain Bristol N2 resulted in no apparent phenotype (Table 1). The same result has been reported by others in the WormBase Database using wild-type non RNAi hypersensitive strains. Based on this observation, it was possible to ask using combinatorial RNAi in a wild-type strain whether other non-essential pre-mRNA processing factors interact genetically with CeRED120.

Previously, we have shown that individual RNAi of CeSRm160 (*rsr-1*), or of CeRNPS1 (*Cernp-5*), both components of the EJC complex, also do not result in an apparent phenotype [47,48]. By contrast, RNAi-mediated depletion of CeY14 (*Cernp-4*), another component of the EJC, resulted in embryonic lethality [47]. The *C. elegans* genome encodes three members of the REF/Aly family, that are believed to function as adaptor proteins mediating the recruitment of the mRNA export factor NXF1/TAP to specific mRNAs in mammalian cells. RNAi of individual REF genes, or co-depletion of all REF genes resulted in an apparent wild-type phenotype, or only caused a minor defect in larval mobility, respectively, and without affecting mRNA export [47]. Similarly, co-depletion by RNAi of CeRED120 and SRm160, RNPS1 or the REF family proteins also does not result in an apparent altered phenotype (Table 1).

In previous studies, we also found that co-depletion by RNAi of CeSRm160 and any one of the CeSR family proteins resulted in a specific defect, leading to the production of unfertilized oocytes in the injected animal [48]. These results provided evidence for the existence of conserved interactions between SRm160 and multiple SR family proteins required for proper development. Interactions between SRm160 and SR family proteins had also been shown to function in the stimulation of both constitutive and enhancer-dependent splicing in the mammalian system. Moreover, we also observed developmental defects when CeSRm160 was co-suppressed with specific 3′-end cleavage factors, consistent with our evidence for function of SRm160 in the stimulation of 3′-end processing in mammalian cells [28,54].

In the present study, we found that simultaneous depletion by RNAi of CeRED120 and CeSRp40 or CeSC35 did not lead to an apparent altered phenotype. By contrast, co-depletion of CeRED120 and CeSRp20 resulted in a slow growth phenotype, with approximately an 8–10 h developmental delay (Table 1). Noticeably, at day 4 post-injection only 20% of the population were adults, whereas 100% of the population were adults following co-depletion of CeRED120 and other CeSR proteins. Thus, consistent with our observation that hRED120 is not required for the splicing of the same substrates as SRm160, these proteins also appear to engage in different genetic interactions involving SR family proteins in the context of whole organism biology in *C. elegans*. Moreover, the observation that simultaneous depletion of CeRED120 and CeSRp20 results in a distinctive phenotype is indicative of important functional interactions between these factors.

Due to the similarity between RED120 and SRm160, which was shown to have a conserved role in 3′-end processing [54], we also investigated possible genetic interactions between RED120 and 3′ end processing factors. RNAi-mediated co-depletion of RED120 and CFIm68 resulted in no apparent phenotype. However, co-depletion of RED120 and Clp1, a subunit of cleavage factor II, resulted in early embryonic lethality (Table 1). Embryos were arrested in development prior to organogenesis, indicating a genetic interaction between RED120 and this component of the cleavage machinery (Table 1 and Fig. 5A). By contrast, we previously reported that co-depletion of SRm160 and Clp1 did not result in an apparent phenotype [54]. Finally, we found that RNAi-mediated co-depletion of RED120 and CstF50 resulted in slow growth and aberrant oogenesis. (Fig. 5B), suggesting a strong genetic interaction of these factors. Compared to wild-type adults, in worms depleted of RED120 and CstF50, the gonad remains syncytial with no mature oocytes forming. Interestingly, we have shown previously that co-depletion of SRm160 and CstF50 leads to late embryonic lethality [54]. This suggests that RED120, may have an important function in 3′-end processing. Altogether, these results are consistent with a conserved role of RED120 in the coupling of splicing and 3′-end formation.

4. Discussion

In this report, we have identified and characterized hRED120, a SRm160 splicing coactivator-interacting protein that assembles into splicing complexes and remains bound to spliced mRNA. hRED120 belongs to a family of proteins that are quite well-conserved from yeast to human. These proteins share several motif features, including a proline rich region and/or an RRM at the amino-terminus, a central domain rich in RE/RD dipeptides, and a PWI nucleic acid binding motif or related domain at the carboxyl-terminus. The motif features of RED120, as well as the observations that it associates with splicing components and functional splicing complexes, concentrates in nuclear speckles enriched in other splicing components in vivo, and forms genetic interactions with both splicing factor and with 3′-end cleavage factor orthologs in *C. elegans*, suggests that it may participate in splicing and/or the coupling of splicing and 3′-end formation.

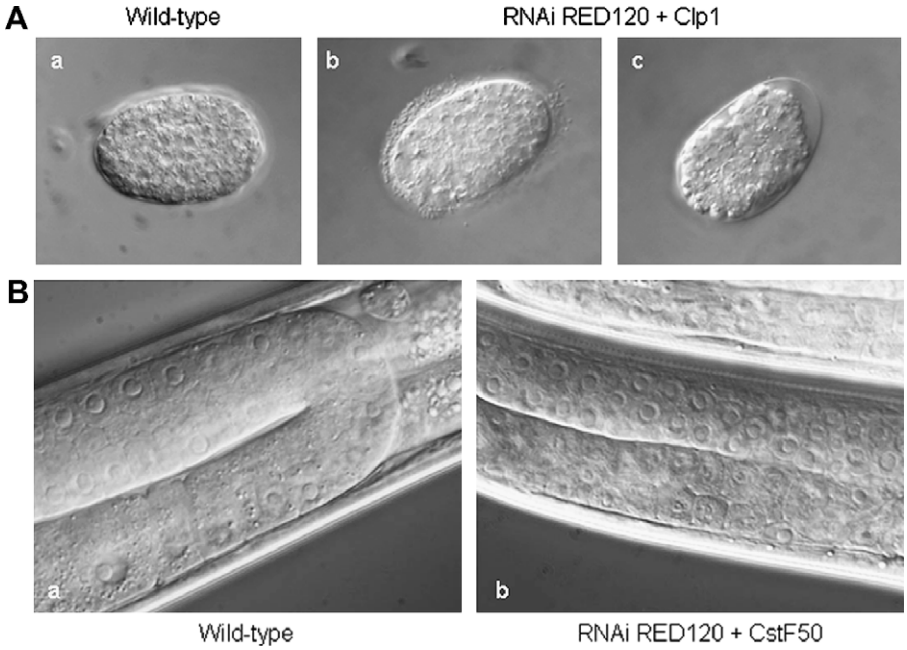

Fig. 5. Interactions between RED120 and components of the 3′end processing machinery are essential for *C. elegans* development. (A) RNAi-mediated codepletion of RED120 and Clp1, a subunit of cleavage factor II, results in early embryonic lethality. Embryonic development is arrested prior organogenesis (panels b and c). Panel a shows wt embryo in corresponding stage. Each embryo is ~50 μm long. (B) RNAi-mediated codepletion of RED120 and cleavage stimulation factor CstF50 results in slow growth and aberrant oogenesis. Panel a shows wt adult gonad with progressively developing oocytes. In worms depleted of RED120 and CstF50 gonad remains syncytial with no detectable oocytes (panel b).

Consistent with these activities, hRED120 was previously identified (as fSAP94) by mass-spectrometry of isolated spliceosomal complexes [55]. Similarly, 2 peptides from RED120 have been identified by mass-spectrometry-based analysis of purified EJC complexes, suggesting that RED120 co-purifies with the EJC complex (Tange and Moore, personal communication). We performed immunoprecipitations with αchRED120 antibody to determine if RED120 binds spliced RNA −20 to −24 nucleotides upstream of the mRNA exon–exon junction, which is where EJC components associate [18]. Although immunoprecipitation of this RNA fragment was detected, additional fragments were also detected, indicating that RED120 may not specifically associate with spliced mRNA at the EJC "docking site" (our unpublished observations). It is also possible that RED120 is weakly associated with the EJC, such that only minor levels of the −24 to −20 protected fragment are observed. In any case, our observation that RED120 associates with multiple EJC components, and the observation that RED120 peptides can be detected in purified EJC complexes, is consistent with a possible weak or transient association between RED120 and one or more EJC components bound to spliced mRNA.

The observations in the present work indicating that hRED120 associates with splicing factors that bind to pre-mRNA at early steps in spliceosome formation, including snRNPs and SRm160, suggest that it may also have a role in splicing. That the association of hRED120 with SRm160 and Sm antigens was resistant to ribonuclease treatments (Figs. 2B and 3C), further suggests that regions in the hRED120 including the RE/RD- and P-rich domains could participate in interactions with other splicing factors. During the assembly of the spliceosome, hRED120 may interact with pre-mRNA via one or more protein-protein interactions and/or via direct interactions mediated by its RRM or PWI motif. Regardless of the mechanism by which hRED120 assembles into splicing complexes, its association with different pre-mRNA substrates and products is specific, since it does not associate with non-splicing-substrates such as U3 snoRNA, or with cDNA-derived mRNAs incubated in splicing reactions.

Immunodepletion of RED120 to ~1% of its endogenous level in HeLa nuclear splicing extract did not alter the splicing activity of four different pre-mRNAs tested (Supplementary information and data not shown). Also, no differences in the efficiency of 3′-end cleavage were detected between hRED120-depleted and mock-depleted extracts (data not shown). This indicates that hRED120 is not a general splicing or 3′-end cleavage factor. Instead, similar to SRm160, RED120 could participate in the constitutive or alternative splicing of specific pre-mRNA substrates. It could also function in the 3′-end processing of specific substrates, possibly via coupled splicing. The identification of specific substrates of RED120, and the elucidation of its mechanism of action represent important goals for future studies.

Our observation of a slow growth defect in *C. elegans* following the simultaneous depletion by siRNA of CeRED120 and CeSRp20, but not after simultaneous depletion of CeRED120 and other splicing components, is consistent with a possible specialized function of RED120 in pre-mRNA splicing, or potentially another step in pre-mRNA metabolism that involves these factors. The developmental defects or the lethality associated with the co-depletion of RED120 and CstF50 or Clp1, suggest an essential role for RED120 in the 3′-end processing of specific mRNAs when the cleavage and polyadenylation machinery is altered.

This proposed function is consistent with the results in the present study demonstrating an interaction between SRm160

and hRED120, the results of our previous study showing that co-depletion of CstF50 and SRm160 in *C. elegans* also causes a late embryonic developmental arrest [54], and our previous results indicating a function for SRm160 in the coupling of splicing and 3′-end cleavage (see Section 1). Taken together, the combined observations suggest that RED120 and SRm160 could form similar interaction networks that function in the coupling of splicing and 3′-end formation. Moreover, the presence in RED120 of a C-terminal PWI nucleic acid binding domain (which is required for SRm160-dependent stimulation of 3′-end cleavage) and N-terminal RRM motifs, separated by a potentially flexible, low complexity region rich in Arg/Glu/Asp residues, makes hRED120 an attractive candidate for linking different RNA molecules or RNP complexes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.066.

References

[1] Jurica, M.S. and Moore, M.J. (2003) Pre-mRNA splicing: awash in a sea of proteins. Mol. Cell 12, 5–14.
[2] Kramer, A. (1996) The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem. 65, 367–409.
[3] Will, C.L. and Luhrmann, R. (1997) Protein functions in pre-mRNA splicing. Curr. Opin. Cell. Biol. 9, 320–328.
[4] Blencowe, B.J., Bowman, A.L., McCracken, S. and Rosonina, E. (1999) SR-related proteins and the processing of messenger RNA precursors. Biochem. Cell Biol. 77, 277–291.
[5] Caceres, J.F. and Kornblihtt, A.R. (2002) Alternative splicing: multiple control mechanisms and involvement in human disease. Trends Genet. 18, 186–193.
[6] Fu, X.-D. (1995) The superfamily of arginine/serine-rich splicing factors. RNA 1, 663–680.
[7] Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. RNA 6, 1197–1211.
[8] Maniatis, T. and Reed, R. (2002) An extensive network of coupling among gene expression machines. Nature 416, 499–506.
[9] Minvielle-Sebastia, L. and Keller, W. (1999) mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. Curr. Opin. Cell Biol. 11, 352–357.
[10] Gilmartin, G.M. (2005) Eukaryotic mRNA 3′ processing: a common means to different ends. Genes Dev. 19, 2517–2521.
[11] Proudfoot, N. and O'Sullivan, J. (2002) Polyadenylation: a tail of two complexes. Curr. Biol. 12, R855–R857.
[12] Kyburz, A., Friedlein, A., Langen, H. and Keller, W. (2006) Direct interactions between subunits of CPSF and the U2 snRNP contribute to the coupling of pre-mRNA 3′ end processing and splicing. Mol. Cell. 23, 195–205.
[13] Millevoi, S., Loulergue, C., Dettwiler, S., Karaa, S.Z., Keller, W., Antoniou, M. and Vagner, S. (2006) An interaction between U2AF 65 and CF I(m) links the splicing and 3′ end processing machineries. EMBO J. 25, 4854–4864.
[14] Vagner, S., Vagner, C. and Mattaj, I.W. (2000) The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3′-end processing and splicing. Genes Dev. 14, 403–413.
[15] Hirose, Y. and Manley, J.L. (2000) RNA polymerase II and the integration of nuclear events. Genes Dev. 14, 1415–1429.
[16] Tange, T.O., Nott, A. and Moore, M.J. (2004) The ever-increasing complexities of the exon junction complex. Curr. Opin. Cell Biol. 16, 279–284.
[17] Kataoka, N., Yong, J., Kim, V.N., Velazquez, F., Perkinson, R.A., Wang, F. and Dreyfuss, G. (2000) Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. Mol. Cell 6, 673–682.
[18] Le Hir, H., Izaurralde, E., Maquat, L.E. and Moore, M.J. (2000) The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions. EMBO J. 19, 6860–6869.
[19] Le Hir, H., Moore, M.J. and Maquat, L.E. (2000) Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon–exon junctions. Genes Dev. 14, 1098–1108.
[20] Kim, V.N., Kataoka, N. and Dreyfuss, G. (2001) Role of the nonsense-mediated decay factor hUpf3 in the splicing- dependent exon–exon junction complex. Science 293, 1832–1836.
[21] Le Hir, H., Gatfield, D., Izaurralde, E. and Moore, M.J. (2001) The exon–exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J. 20, 4987–4997.
[22] Lykke-Andersen, J. (2001) mRNA quality control: marking the message for life or death. Curr. Biol. 11, R88–R91.
[23] Nott, A., Le Hir, H. and Moore, M.J. (2004) Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. Genes Dev. 18, 210–222.
[24] Tange, T.O., Shibuya, T., Jurica, M.S. and Moore, M.J. (2005) Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. RNA 11, 1869–1883.
[25] Blencowe, B.J., Issner, R., Nickerson, J.A. and Sharp, P.A. (1998) A coactivator of pre-mRNA splicing. Genes Dev. 12, 996–1009.
[26] Eldridge, A.G., Li, Y., Sharp, P.A. and Blencowe, B.J. (1999) The SRm160/300 splicing coactivator is required for exon-enhancer function. Proc. Natl. Acad. Sci. USA 96, 6125–6130.
[27] McCracken, S., Lambermon, M. and Blencowe, B.J. (2002) SRm160 splicing coactivator promotes transcript 3′-end cleavage. Mol. Cell Biol. 22, 148–160.
[28] Szymczyna, B.R. et al. (2003) Structure and function of the PWI motif: a novel nucleic acid-binding domain that facilitates pre-mRNA processing. Genes Dev. 17, 461–475.
[29] Blencowe, B.J., Nickerson, J.A., Issner, R., Penman, S. and Sharp, P.A. (1994) Association of nuclear matrix antigens with exon-containing splicing complexes. J. Cell Biol. 127, 593–607.
[30] Wan, K., Nickerson, J.A., Krockmalnic, G. and Penman, S. (1994) The B1C8 protein is in the dense assemblies of the nuclear matrix and relocates to the spindle and pericentriolar filaments at mitosis. Proc. Natl. Acad. Sci. USA 91, 594–598.
[31] Blencowe, B.J., Bauren, G., Eldridge, A.G., Issner, R., Nickerson, J.A., Rosonina, E. and Sharp, P.A. (2000) The SRm160/300 splicing coactivator subunits. RNA 6, 111–120.
[32] Fabrizio, P., Laggerbauer, B., Lauber, J., Lane, W.S. and Luhrmann, R. (1997) An evolutionarily conserved U5 snRNP-specific protein is a GTP-binding factor closely related to the ribosomal translocase EF-2. EMBO J. 16, 4092–4106.
[33] Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E. and Mattaj, I.W. (1994) A nuclear cap binding protein complex involved in pre-mRNA splicing. Cell 78, 657–668.
[34] Trembley, J.H., Hu, D., Hsu, L.C., Yeung, C.Y., Slaughter, C., Lahti, J.M. and Kidd, V.J. (2002) PITSLRE p110 protein kinases associate with transcription complexes and affect their activity. J. Biol. Chem. 277, 2589–2596.

[35] Gatfield, D. and Izaurralde, E. (2002) REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export. J. Cell Biol. 159, 579–588.
[36] Lerner, E.A., Lerner, M.R., Janeway Jr., C.A. and Steitz, J.A. (1981) Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc. Natl. Acad. Sci. USA 78, 2737–2741.
[37] Fu, X.D. and Maniatis, T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature 343, 437–441.
[38] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[39] Blencowe, B.J. and Ouzounis, C.A. (1999) The PWI motif: a new protein domain in splicing factors. Trends Biochem. Sci. 24, 179–180.
[40] Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C.J., Hofmann, K. and Bairoch, A. (2002) The PROSITE database, its status in 2002. Nucleic Acids Res. 30, 235–238.
[41] Wootton, J.C. and Federhen, S. (1996) Analysis of compositionally biased regions in sequence databases. Methods Enzymol. 266, 554–571.
[42] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
[43] Fortes, P., Lamond, A.I. and Ortin, J. (1995) Influenza virus NS1 protein alters the subnuclear localization of cellular splicing components. J. Gen. Virol. 76 (Pt 4), 1001–1107.
[44] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475–1489.
[45] Fortes, P., Bilbao-Cortes, D., Fornerod, M., Rigaut, G., Raymond, W., Seraphin, B. and Mattaj, I.W. (1999) Luc7p, a novel yeast U1 snRNP protein with a role in 5′ splice site recognition. Genes Dev. 13, 2425–2438.
[46] Hamm, J., Dathan, N.A. and Mattaj, I.W. (1989) Functional analysis of mutant Xenopus U2 snRNAs. Cell 59, 159–169.
[47] Longman, D., Johnstone, I.L. and Caceres, J.F. (2003) The Ref/Aly proteins are dispensable for mRNA export and development in *Caenorhabditis elegans*. RNA 9, 881–891.
[48] Longman, D., McGarvey, T., McCracken, S., Johnstone, I.L., Blencowe, B.J. and Caceres, J.F. (2001) Multiple interactions between SRm160 and SR family proteins in enhancer-dependent splicing and development of *C. elegans*. Curr. Biol. 11, 1923–1933.
[49] McCracken, S. et al. (2005) Proteomic analysis of SRm160-containing complexes reveals a conserved association with cohesin. J. Biol. Chem. 280, 42227–42236.
[50] Sherrington, R. et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375, 754–760.
[51] Gottschalk, A. et al. (1998) A comprehensive biochemical and genetic analysis of the yeast U1 snRNP reveals five novel proteins. RNA 4, 374–393.
[52] Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. 17, 1030–1032.
[53] Fortes, P., Kufel, J., Fornerod, M., Polycarpou-Schwarz, M., Lafontaine, D., Tollervey, D. and Mattaj, I.W. (1999) Genetic and physical interactions involving the yeast nuclear cap-binding complex. Mol. Cell Biol. 19, 6543–6553.
[54] McCracken, S., Longman, D., Johnstone, I.L., Caceres, J.F. and Blencowe, B.J. (2003) An evolutionarily conserved role for SRm160 in 3′-end processing that functions independently of exon junction complex formation. J. Biol. Chem. 278, 44153–44160.
[55] Zhou, Z., Licklider, L.J., Gygi, S.P. and Reed, R. (2002) Comprehensive proteomic analysis of the human spliceosome. Nature 419, 182–185.