

hLodestar/HuF2 interacts with CDC5L and is involved in pre-mRNA splicing

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Abstract

hLodestar/HuF2 belongs to the SNF2 family of proteins. This family of proteins has been shown to play a critical role in altering protein–DNA interactions in a variety of cellular contexts. We have identified an unexpected interaction between hLodestar/HuF2 and CDC5L in both the yeast two-hybrid system and HeLa nuclear extract. CDC5L is a well-characterized pre-mRNA splicing factor in yeast and humans. Our findings demonstrate that hLodestar/HuF2 associates with human splicing complexes. We also found that a truncated hLodestar/HuF2 polypeptide that overlaps with the CDC5L-binding region can inhibit pre-mRNA splicing by disrupting spliceosome assembly. These findings indicate that hLodestar/HuF2 may have a role in pre-mRNA splicing. These data are consistent with a close co-ordination of the transcription and splicing pathways in eukaryotes. Although many members of the DExH/D helicase superfamily have been linked to pre-mRNA splicing, this is the first SNF2 family member to be implicated in this pathway.

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The SNF2 family is a conserved group of proteins found throughout evolution that participate in a number of different cellular activities [1]. This family is named after the yeast *Saccharomyces cerevisiae* transcriptional activator protein SNF2. The SNF2 general activation complex was found to perform a nucleosomal disruption function in the yeast RNA polymerase II holoenzyme, facilitating binding of activation factors [2]. These ATP-dependent-remodeling complexes use ATP hydrolysis to increase the accessibility of DNA associated with nucleosomes and are now commonly referred to as ATP-dependent chromatin-remodeling complexes. It is well established that these chromatin-remodeling complexes can alter the positions of nucleosomes along DNA in vitro. In addition, the human and yeast SWI/SNF complexes can introduce topological changes in closed circular nucleosomal arrays (reviewed in [3]), and the CHARD complex from *Drosophila* can alter the spacing

between nucleosomes [4]. However, it is not yet clear whether these same mechanisms are used in vivo.

In addition to transcriptional activation by SNF2, transcriptional repression by Mot1 also is represented in the SNF2 protein family. Auble et al. [5] have suggested that the SNF2 motifs encode an ATPase-dependent conserved function, which is believed to be a disruption of protein–DNA complexes that occurs during chromatin remodeling.

Based on conserved sequence motifs, the human factor 2 (HuF2) protein belongs to the SNF2 protein family. *Drosophila* factor 2 (DmF2) was originally identified in purified fractions of *Drosophila* K_c cell nuclear extracts by its ability to suppress the generation of long RNA polymerase II transcripts [6]. Later, this isolated protein activity was found to cause the release of transcripts by RNA polymerase II in an ATP-dependent manner [7]. Eventually, DmF2 was determined to be identical to the *Drosophila lodestar* protein. The human homolog of this protein (hLodestar/HuF2) was cloned and studied in comparison to the *Drosophila lodestar* activities. The results of these studies show that, similar to the

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Drosophila protein, hLodestar/HuF2 has dsDNA-dependent ATPase and transcription termination activity in vitro [8]. This dsDNA-dependency suggests that the double helical structure of DNA is essential for ATPase activity. HuF2 also has been shown to release RNA polymerase I or II that is stalled at a cyclobutane thymine dimer [9]. *Lodestar* was originally identified as a maternal-effect gene essential for embryonic mitosis in *Drosophila* [10]. Mutations in *lodestar* were shown to cause chromatin bridging during anaphase. Tangled and sometimes fragmented anaphase chromosomes were found at the metaphase plate in embryos that do not contain a wild-type copy of *lodestar*.

The *Schizosaccharomyces pombe* *cdc5⁺* gene was originally identified in a screen for fission yeast mutants defective for cell cycle progression [11]. Work in other organisms has revealed that *cdc5⁺* is highly conserved and possible orthologs have been identified in *Arabidopsis thaliana*, *S. cerevisiae* (CEF1), *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, and human (CDC5L). CDC5L proteins are essential for G2/M progression in *S. pombe* and *S. cerevisiae* [12,13], and these proteins contain homology to the DNA-binding domain of human c-Myb [12]. In contrast to c-Myb, however, CDC5L proteins contain only two repeats of the helix-turn-helix motif, whereas Myb family members possess three repeats. The Myb domains of CDC5L proteins have shown affinity for double-stranded DNA [12,14]. In addition, the region of CDC5L containing the Myb motifs has been shown to bind specifically to a 12-bp DNA sequence. This DNA–protein interaction was found to be capable of activating transcription [15].

More recently, CDC5L has been investigated for its association with the spliceosome and for potential roles it may play in pre-mRNA splicing. CDC5L was first identified as a spliceosomal protein using 2D protein gel electrophoresis and mass spectrometric analysis of purified human splicing complexes [16]. Since then, several other studies have been carried out to analyze the function of this protein in pre-mRNA splicing. These studies were performed using yeast and mammalian cells [17–19].

In this report we show that hLodestar/HuF2 interacts with CDC5L and that it is a component of the human spliceosome complex. We also found that a truncated form of the human hLodestar/HuF2 protein inhibits pre-mRNA splicing in vitro, presumably by interfering with spliceosome assembly. This report therefore provides the first evidence that an SNF2 family member may also have a role in pre-mRNA splicing.

Materials and methods

DNA cloning and sequencing. The DNA sequence of both strands of hLodestar/HuF2 cDNA was determined with the ABI-PRISM model 377 automated sequencer (Perkin Elmer, Foster City, CA). Oligonu-

cleotides used in DNA sequencing and PCR were designed with the Oligo Primer Analysis Program (National Biosciences, Plymouth, MN). Nucleotides 447–1358 were cloned into the pET28a expression vector (Novagen, Madison, WI) and the resulting construct was termed tr-hLdstr.

Protein expression and antibody preparation. The tr-hLdstr construct was expressed in BL21 (DE3) *Escherichia coli* [20]. The His-tagged protein was purified with Ni–NTA–agarose (Qiagen, Chatsworth, CA), following the manufacturer's protocols. The purified protein was used to raise polyclonal antibodies in rabbits (Alpha Diagnostic International, San Antonio, TX). We generated an affinity column to purify anti-hLodestar/HuF2 antibody using an Amino-link Kit (Pierce, Rockford, IL), according to the manufacturer's recommendations. Antibody binding and elution were performed as described previously [21]. Eluted fractions were combined and dialyzed against PBS with 0.02% sodium azide.

Immunoprecipitation. Purified anti-hLodestar/HuF2 antibodies were incubated with either 40 µg of HeLa whole-cell extract or 100 µg of HT1080 [³⁵S]methionine-labeled cell lysates on ice for 1 h. Cells were labeled as described by Harlow and Lane [21]. Reactions were added to Sepharose A beads that had been washed with NP-40 lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM Tris, pH 8.0) and incubated for an additional hour at 4°C with rotation. Beads and bound protein were then washed three times with NP-40 lysis buffer, resuspended in 30 µl 2× SDS buffer, and boiled for 5 min and were then separated by SDS–PAGE.

For large-scale immunoprecipitation, 50 mg of HeLa nuclear extract was incubated with 150 µg of affinity-purified anti-hLodestar/HuF2 antibodies or pre-immune serum on ice for 2 h. The reaction was then added to 100 µl of NP-40 buffer-washed protein A–Sepharose beads and rotated at 4°C for 2 h. Beads and bound proteins were then washed five times in NP-40 lysis buffer, resuspended in 80 µl 2× SDS buffer, and boiled for 5 min. Samples were separated by SDS–PAGE and stained with colloidal Coomassie blue, according to the manufacturer's instructions (Sigma). Bands that were unique to the anti-hLodestar/HuF2 sample were extracted and sequenced with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

SDS–PAGE and Western blotting. SDS–PAGE gel analysis and Western blotting were performed as described previously [22,23]. For Western blotting, we used the following primary antibodies: 5P10 monoclonal anti-p80 coilin antibody (1:100); anti-CDC5L antibodies (1:1000); and anti-hLodestar antibody (1:2000). Protein bands were detected through appropriate peroxidase-conjugated secondary antibodies (Pierce or Amersham–Pharmacia) and blots were developed with the ECL kit (Amersham–Pharmacia) according to the manufacturer's instructions.

Yeast two-hybrid screening. A human lymphocyte cDNA library fused to the GAL4 activation domain of the pACT vector (gift from Stephen Elledge) was screened with pAS2.1-5'hLdstr as bait in the yeast strain Y190 [24]. pAS2.1-5'hLdstr was prepared by subcloning the region encompassing amino acids 1–188 of hLodestar/HuF2 into the yeast two-hybrid bait vector pAS2.1. Library transformations were performed as described previously using a high-efficiency lithium acetate method [25]. Positive clones were confirmed by transferring them to the yeast strain PJ69-A4, which contained the pAS2.1-5'hLdstr, to test for activation of the reporter genes ADE2, HIS3, and lacZ under the GAL2, GAL1, and GAL7 promoters, respectively [26]. These colonies were tested for β-galactosidase activity with an X-gal overlay assay.

Splicing assay. [³²P] uniformly labeled pre-mRNA was prepared in vitro from the plasmid pBSAd1 [27]. The plasmid was linearized with Sau3AI and purified by successive phenol/chloroform extractions and ethanol precipitation. Approximately 2 µg of digested plasmid was used for in vitro transcription, as described previously [27], using the enzyme T3 RNA polymerase (Promega, Madison, WI). Splicing assays were performed with uniformly labeled capped pre-mRNAs incubated with nuclear extract, as described previously [28]. Nuclear extracts used

in the splicing assays were obtained from Computer Cell Culture Centre (Mons, Belgium). When the reactions were to be used to analyze splicing complexes, they were loaded onto a polyacrylamide/agarose composite gel and run for 5 h at 25 mA [27]. The spliceosome complex was purified from splicing reactions using standard methods involving a two-step process, i.e., gel filtration and affinity chromatography, as described previously [16].

Results

Characterization of hLodestar/HuF2 antibodies

To initiate the study of identifying and characterizing the interacting partners of hLodestar/HuF2 (Fig. 1A) we raised rabbit antiserum and prepared affinity-purified antibodies to the protein using a bacterially expressed fragment of the protein (tr-hLdstr). The purified anti-hLodestar/HuF2 antibody preparation was characterized by testing its ability to specifically recognize the bacterially expressed truncated protein (Fig. 1B, lanes 2–4) and endogenous hLodestar/HuF2 in mammalian cell lysates on an immunoblot. We were unable to express the full-length hLodestar/HuF2 protein in *E. coli* because of problems with degradation presumably due to the protein's size and/or toxicity to bacteria. A protein band of the appropriate size was present on the Western blot (Fig. 1B, lane 1) when HT1080 cell lysates were used in Western blot analysis. The hLodestar/HuF2 protein labeled in vitro using [³⁵S]methionine by coupled transcription–translation also produced a band of similar size that was readily immunoprecipitated with the

affinity-purified antibodies (data not shown). These results indicate that the anti-hLodestar/HuF2 antibodies will recognize specifically the bacterially expressed truncated hLodestar/HuF2 polypeptide as well as the endogenous protein.

Identifying functional protein–protein interactions of hLodestar/HuF2

To identify proteins that interact with hLodestar/HuF2 in vivo, we used the yeast two-hybrid system [23,29,30] to determine the cellular partners of hLodestar/HuF2. A region encompassing the 5' region hLodestar/HuF2 cDNA was transferred into the yeast two-hybrid bait vector pAS2.1 to produce pAS2.1-5'hLdstr. The pAS2.1-5'hLdstr construct tested negative for self-activation in the yeast two-hybrid system and was subsequently used to screen more than 100 million clones with the Y190 yeast strain. Restriction enzyme and Southern blotting analyses identified 30 unique clones. After the 5' ends of all clones were sequenced, the number of unique clones was reduced to 17. These clones were transferred to the yeast strain PJ69-A4 to test for activation of the reporter genes ADE2, HIS3, and lacZ under the GAL2, GAL1, and GAL7 promoters, respectively. In contrast to the Y190 strain, the reporter genes in PJ69-A4 utilize different inducible promoters that reduce false positives due to promoter binding and yet utilize the same interaction mechanism of the GAL4 DNA binding and activation domains [25]. This final screen produced 10 positive clones (Table 1).

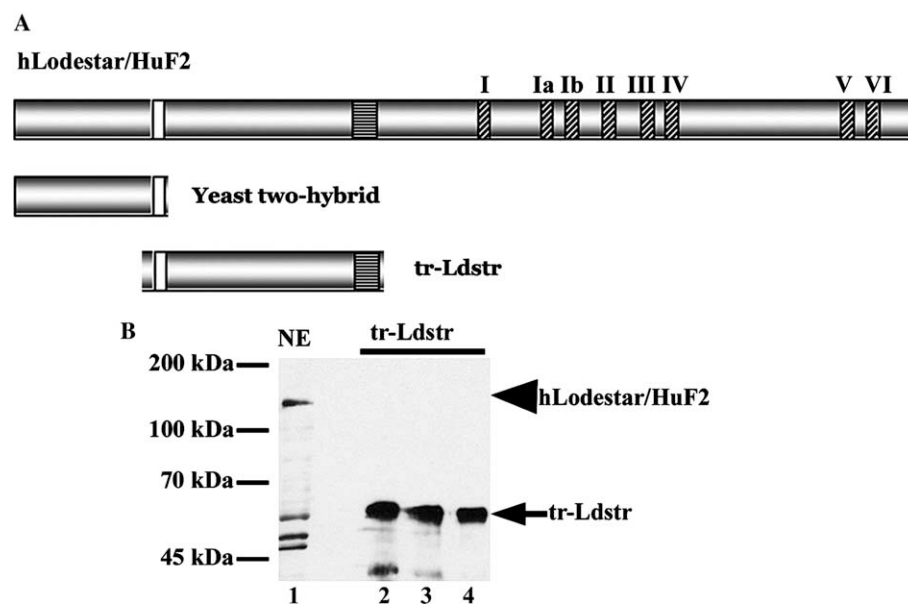


Fig. 1. Schematic representation of hLodestar/HuF2 domain structure. (A) Full-length hLodestar/HuF2 and constructs used in this study. The open box represents a putative nuclear localization signal. The box shaded with horizontal lines represents a leucine zipper motif and the boxes labeled and filled with diagonal lines represent the conserved SNF2 domains. (B) Western blot analysis using purified anti-hLodestar/HuF2. Lane 1 contains 25 μ g HeLa nuclear extract and lanes 2–4 contain 10, 5, and 1 ng tr-hLdstr recombinant protein, respectively.

Table 1
Proteins interacting with hLodestar/HuF2 in yeast two-hybrid system

Protein	Swiss protein Accession No.	Mol. wt. (kDa)	Comments
CDC5L	Q99974	92.5	Spliceosomal protein, 2 Myb like domains
TACC3	Q9Y6A5	90.3	Coiled coil, may contrib. to cancer
SKD3 (SLPA/CLPB family)	Q8H078	78.7	4 ANK repeats CLPA/CLPB family
Nef-associated factor 1	Q15025	71.8	Inhibits TNF-induced NF- κ -B-dependent gene expression
ALG-2-interacting protein 1	Q9UKL5	96.0	Contains proline rich –COOH terminal domain
B-myb	P10244	78.7	3 Myb domains, phosphorylated in S phase
NC2 protein	Q14919	22.3	TATA binding
Nuclear VCP-like protein NVLP.2	O15381	95	Contains AAA domain, ATP binding
SMIF TF	Q9NPI6	63.3	Transcription factor
Peroxisredoxin 4	Q13162	30.5	Redox regulation

In a second approach we used co-immunoprecipitation to verify the positive protein–protein interactions revealed in the yeast two-hybrid screening, as well as to detect additional interacting partners to hLodestar/HuF2. To determine the feasibility of co-immunoprecipitation, we first performed these experiments with anti-hLodestar/HuF2 antibodies on [³⁵S]methionine-labeled HT1080 cell lysates. The pre-immune serum was used as the control in this experiment in order to eliminate non-specific bands. Several specific bands that were not present in the control lane were observed in the sample co-immunoprecipitated with anti-hLodestar/HuF2 antibodies (Fig. 2A, lane 2). To identify the specifically co-immunoprecipitated proteins by mass spectrometry, we performed a large-scale co-immunoprecipitation. Specific bands in the anti-hLodestar/HuF2 antibody immunoprecipitation experiment (Fig. 2A, lane 2), compared to the pre-immune control (Fig. 2A, lane 1), were excised and subsequently analyzed by mass spectrometry. Unique proteins identified by mass spectrometry are shown in Table 2. Several of the proteins in Tables 1 and 2 were found either to contain sequence motifs that suggest a function in gene expression, or have been found to be involved in gene transcription/pre-mRNA splicing. These results suggest that hLodestar/HuF2 may have multiple roles in the regulation of gene expression in the cell through interactions with proteins involved in DNA binding, transcription, and pre-mRNA splicing.

A comparison of the two tables shows that CDC5L was detected in both the co-immunoprecipitation experiment and the yeast two-hybrid screen. In order to confirm the association between CDC5L and hLodestar/HuF2 in mammalian cells, we performed a series of co-immunoprecipitation experiments using HeLa nuclear extract. These experiments involved immunoprecipitations with anti-CDC5L, or affinity-purified anti-hLodestar/HuF2 antibodies. The co-immunoprecipitates obtained from these experiments were analyzed by Western blotting (Figs. 2B and C). In the first instance, the immunoprecipitations were performed using

anti-hLodestar/HuF2 antibodies. Immunoprecipitated proteins were separated on an SDS–PAGE gel and analyzed by immunoblotting. The immunoblots were then probed with anti-CDC5L antibodies. Our results from these experiments confirm the observation that anti-hLodestar/HuF2 antibodies will co-immunoprecipitate CDC5L whereas pre-immune IgG does not (Fig. 2B, compare lane 3 with lanes 4 and 5). In a second set of experiments, the immunoprecipitations from HeLa nuclear extracts were done using anti-CDC5L antibodies. Immunoprecipitated proteins were separated on an SDS–PAGE gel and transferred onto a nitrocellulose membrane by electroblotting. The membrane containing the transferred proteins was subsequently probed with anti-hLodestar/HuF2 antibodies. The results obtained from these experiments indicated that anti-CDC5L antibodies will co-immunoprecipitate hLodestar/HuF2 from HeLa nuclear extracts. These data, combined with the yeast two-hybrid results, strongly suggest that hLodestar/HuF2 interacts with the splicing factor CDC5L. Also, the association of hLodestar/HuF2 with other well-known spliceosomal proteins in HeLa cells as shown by the mass spectrometry analysis (Table 2, i.e., hnRNP A1 and hnRNP A2/B) suggests a possible role for the protein in pre-mRNA splicing. For example, hnRNP A1/A2 are known to be required for 5' and 3' splice site selection during pre-mRNA splicing in mammalian cells ([31] and references therein).

The hLodestar/HuF2 mutant (tr-hLdstr) will inhibit in vitro splicing in HeLa nuclear extract

CDC5L is required for efficient pre-mRNA splicing in yeast and human cells [16–19,32]. Immunodepletion of CDC5L from HeLa nuclear extracts inhibits pre-mRNA splicing without blocking the assembly of splicing complexes [19]. Because of the interaction between CDC5L and hLodestar/HuF2 described above, we next investigated whether hLodestar/HuF2 may be involved in the pre-mRNA splicing machinery. The truncated hLodestar/HuF2 (tr-hLdstr) polypeptide that

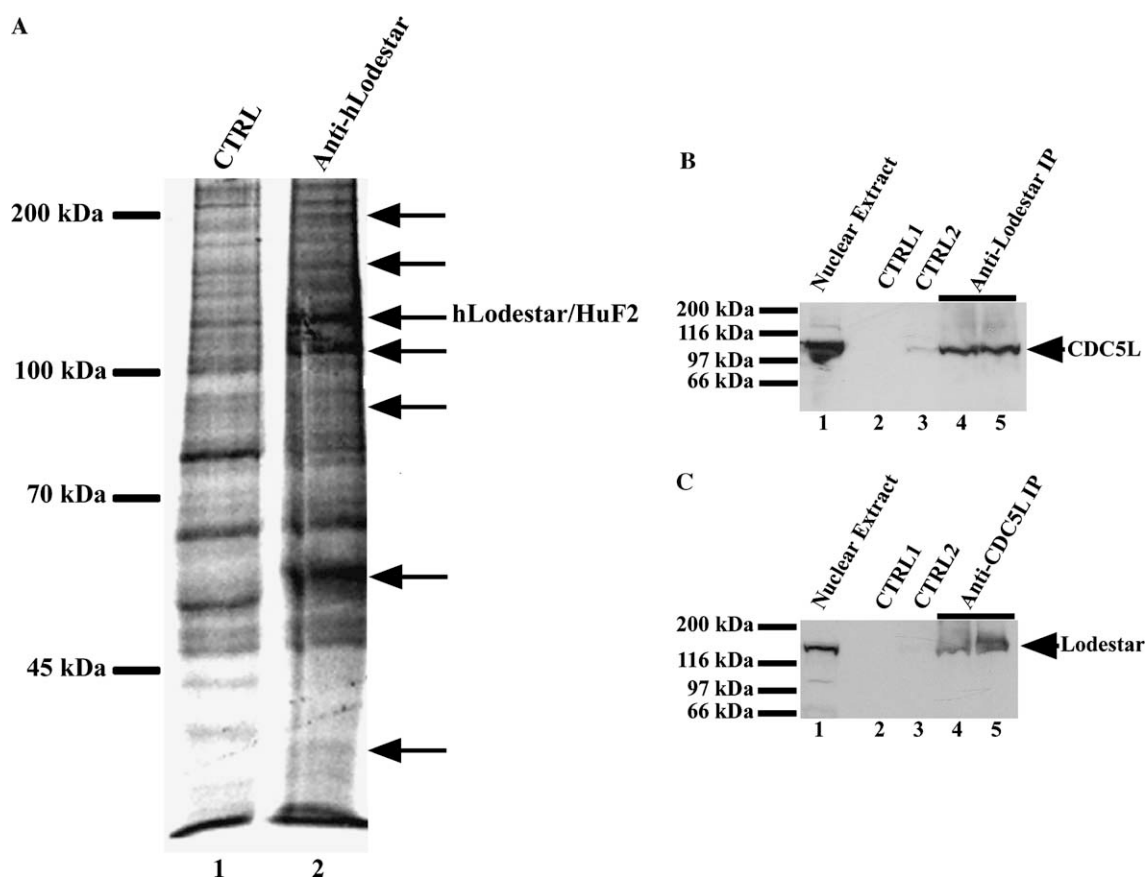


Fig. 2. hLodestar/HuF2 associates with CDC5L in cell lysates and HeLa nuclear extracts. (A) Autoradiograph of anti-hLodestar/HuF2 IP using ^{35}S -labeled HT1080 cell lysates. Lane 1 (CTRL) contains proteins immunoprecipitated using pre-immune sample and lane 2 contains proteins immunoprecipitated by the anti-hLodestar antiserum. Protein markers are shown at the left of each panel. Protein bands absent in the control experiment are shown by the arrows on the right. (B) Anti-hLodestar/HuF2 antibodies co-immunoprecipitate CDC5L from HeLa nuclear extracts. Immunoprecipitations were performed using 0.5 mg of HeLa nuclear extract. Western blot analysis of the immunoprecipitates was done using rabbit anti-hLodestar/HuF2 antibodies and the membrane was probed with sheep anti-CDC5L antibodies. Lane 1 contains HeLa nuclear extract (40 μg) used as a positive control. Lanes 2 and 3 (CTRL1 and CTRL2) contain the immunoprecipitates from the bead and pre-immune controls, respectively. Lanes 4 and 5 contained duplicate immunoprecipitations using hLodestar/HuF2 antibodies. Protein markers are shown on the left of each panel, and the arrowhead on the right of the panel indicates the CDC5L protein band. (C) Anti-CDC5L antibodies co-immunoprecipitate hLodestar/HuF2 from HeLa nuclear extracts. Immunoprecipitations were performed using 0.5 mg of HeLa nuclear extract as above. Western blot analysis of the immunoprecipitates was done using sheep anti-CDC5L antibodies and the protein was transferred onto the nitrocellulose membrane probed with rabbit anti-hLodestar/HuF2 antibodies. Lane 1 contains HeLa nuclear extract used as a positive control. Lanes 2 and 3 (CTRL1 and CTRL2) contain the immunoprecipitates from the bead and pre-immune controls, respectively. Lanes 4 and 5 contained duplicate immunoprecipitations using anti-CDC5L antibodies. Protein markers are shown on the left of each panel, and the arrowhead on the right of the panel shows the hLodestar/HuF2 protein band.

Table 2
Proteins co-immunoprecipitating with hLodestar/HuF2

Protein	Swiss protein Accession No.	Mol. wt. (kDa)	Comments
CDC5L	Q99974	92.5	Spliceosomal protein, 2 Myb like domains
hnRNP A1	P09651	38.7	2 RNA recognition motifs (RRM)
hnRNP A2/B1	P22626	37.4	2 RNA recognition motifs (RRM)
Matrin 3	P43243	94.6	2 RRM and zinc finger domain
Chromokinesin	O95239	139.9	Kinesin motor coiled-coil

overlaps with the CDC5L interacting region of the protein, as determined by the two-hybrid screening, was expressed in *E. coli* and purified by affinity chromatography using an agarose Ni-NTA column (Qiagen). This

region of hLodestar/HuF2 does not contain any of the conserved SNF2 domains present in the protein. The tr-hLdstr sequence is part of the unique region present in hLodestar/HuF2 when compared to other SNF2

domain-containing proteins. The non-conserved region of the polypeptide is thought to confer functional specificity to SNF2 family members. We therefore decided to determine whether the tr-hLdstr polypeptide is capable of blocking the endogenous protein's function by competition. The bacterially expressed protein (tr-hLdstr) was added to *in vitro* splicing reactions to investigate whether the mutant hLodestar/HuF2 will have an effect on splicing. As shown in Fig. 3, lanes 5–7, when tr-hLdstr was added to splicing reactions, splicing of the pre-mRNA substrate was inhibited whereas splicing was unaffected in the control reactions (Fig. 3, lanes 3 and 4). These results indicate that tr-hLdstr may have a dominant-negative effect on the function of hLodestar/HuF2 in HeLa nuclear extracts and suggest a possible role for hLodestar/HuF2 in pre-mRNA splicing through its interaction with CDC5L.

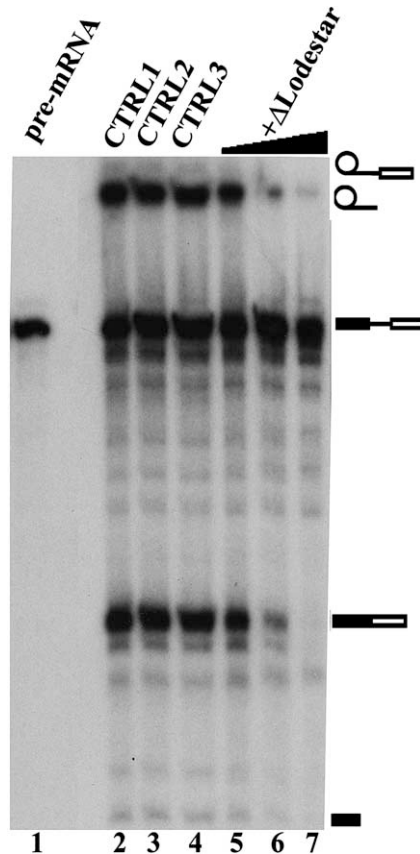


Fig. 3. tr-hLdstr inhibits splicing of pre-mRNA *in vitro*. Bacterially expressed proteins in PBS (0.05–0.2 nmol) were added to about 55 μ g of HeLa nuclear extract and pre-incubated at 30 $^{\circ}$ C for about 15 min before initiating the pre-mRNA splicing reactions. The splicing reactions were incubated at 30 $^{\circ}$ C for about 90 min. Lane 1 contains the pre-mRNA used in the splicing experiments. Lanes 2–4 are control pre-mRNA splicing reactions except that 0.2 nmol of GST and His-tagged CDC5L was added to the reactions in lanes 3 and 4, respectively. Lanes 5–7 represent splicing reactions to which were added increasing amounts of tr-hLdstr (0.05–0.2 nmol). The symbols on the right of the figure represent the input pre-mRNAs, splicing intermediates, and products. The other bands in the figure represent non-specific RNA degradation products.

Effect of tr-hLdstr on spliceosome assembly

We next investigated whether the splicing inhibition observed above was caused by blocking of splicing catalysis or spliceosome assembly. The results from this experiment (Fig. 4) indicate that tr-hLdstr blocks the formation of splicing complexes *in vitro* (Fig. 4, lanes 4–6) whereas spliceosome assembly proceeded normally in the control reactions (Fig. 4, lanes 2 and 3). These results suggest that tr-hLdstr inhibits splicing by interfering with spliceosome assembly and are consistent with a role for the interaction between CDC5L and hLodestar/HuF2 in pre-mRNA splicing.

hLodestar/HuF2 is a stable component of the human spliceosome complex

We next examined whether hLodestar/HuF2 is incorporated into the spliceosome complex assembled *in vitro* since the protein interacts with the splicing factor

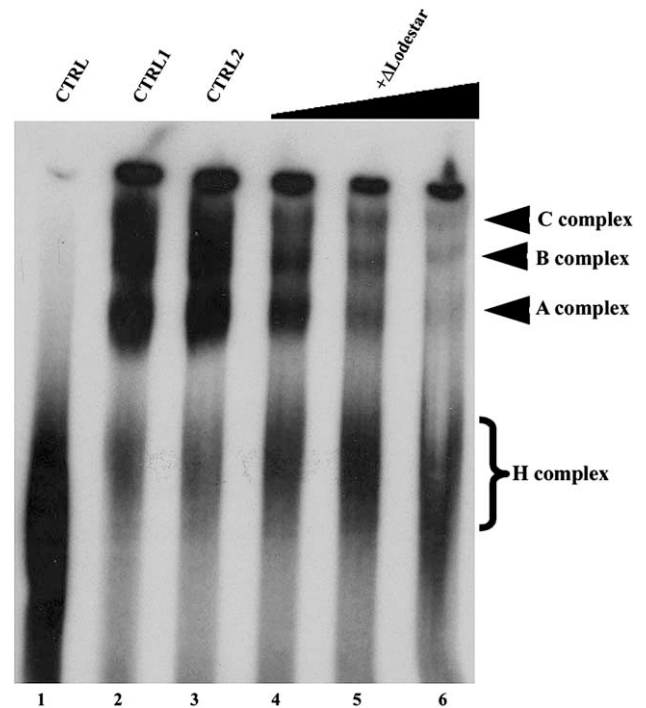


Fig. 4. tr-hLdstr blocks splicing by inhibiting spliceosome assembly. A native polyacrylamide/agarose gel was used to separate spliceosome complexes. The bands on the gel were revealed by autoradiography. Approximately 0.05–0.2 nmol of bacterially expressed protein was added to about 55 μ g of HeLa nuclear extract and pre-incubated at 30 $^{\circ}$ C for 15 min before being added to pre-mRNA splicing reactions as described above, except that these reactions were incubated at 30 $^{\circ}$ C for only 45 min in order to allow for complex assembly before loading onto native gels. Lane 1 contains a control splicing reaction prepared on ice. Lane 2 (CTRL1) is the positive control to which no *E. coli*-expressed protein was added. CTRL2 (lane 3) is a control reaction to which His-tagged CDC5L or GST was added. Lanes 4–6 contain splicing reactions with 0.05, 0.1, and 0.2 nmol of tr-hLdstr, respectively.

CDC5L *in vivo*. The spliceosome complex was purified from splicing reactions using HeLa nuclear extracts under standard conditions that include a two-step process, i.e., gel filtration and affinity chromatography [28,33–37]. We separated the purified spliceosomal proteins by SDS-PAGE and the separated proteins were transferred onto a nitrocellulose membrane by electroblotting. The membrane was then probed with anti-hLodestar/HuF2 antibodies. The presence of hLodestar/HuF2 on the membrane was revealed by enhanced chemiluminescence. As shown in Fig. 5A, lanes 2–4, hLodestar/HuF2 is present in all the three different preparations of purified splicing complexes. Although the yields from the purifications of splicing complexes may vary between preparations, the presence of the specific spliceosomal protein components is highly reproducible as judged by immunoblotting and mass spectrometric analyses (data not shown). We also probed the blot containing the purified splicing complexes with anti-CDC5L antibodies and consistent with previous results [28,35–37], we found that CDC5L is present in all the preparations (Fig. 5B, lanes 2–4). Probing the same blots using antibodies to p80 coilin as a negative control showed that this non-spliceosomal nuclear protein was absent in all the spliceosomal preparations (Fig. 5C, lanes 2–4). These results indicate that hLodestar/HuF2 is a stable component of the human splicing complexes. Taken together, our results suggest a possible novel function for hLodestar/HuF2 in pre-mRNA splicing.

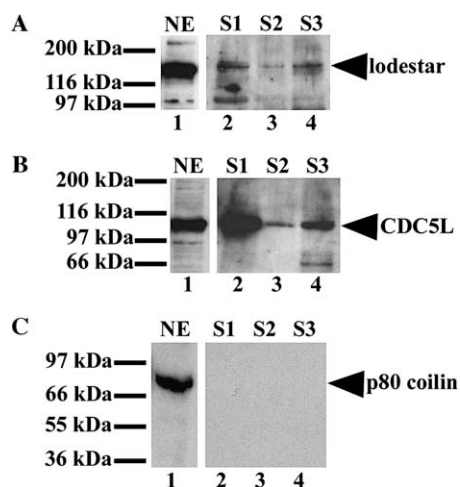


Fig. 5. hLodestar/HuF2 is a stable component of the human splicing complexes. (A) Splicing complexes were purified as described in the Materials and methods section. The proteins in the complexes were separated on a 4–12% SDS-PAGE gradient gel (Invitrogen). The spliceosomal proteins were blotted onto a nitrocellulose membrane before probing with anti-hLodestar/HuF2 antibodies. Bound proteins were identified by enhanced chemiluminescence (ECL). Lane 1 is a control lane containing about 10–15 μ g of total HeLa nuclear extract. Lanes 2–4 contained three independent preparations of purified spliceosomal complexes. (B,C) The contents of the lanes are identical to (A) except that the blots in (B) and (C) were probed with anti-CDC5L and anti-p80 coilin antibodies, respectively.

Discussion

To date, no other SNF2 protein family members have been shown to have a function in pre-mRNA processing. This study has demonstrated that hLodestar/HuF2 interacts with CDC5L and that hLodestar/HuF2 may be involved in efficient spliceosome assembly perhaps through its helicase activity. Although many other members of the DExH/D helicase superfamily have been linked to splicing, this is the first SNF2 family member to be implicated in this pathway. The hLodestar/HuF2-interacting protein CDC5L has been identified by mass spectrometry in several studies as a component of the spliceosome [16,35–37]. hLodestar/HuF2 has not been identified in these studies. However, the non-identification of hLodestar/HuF2 in splicing complexes by mass spectrometry is not unique to this protein because several other proteins that have been shown to be involved in pre-mRNA splicing, e.g., CDC42 [38], ZNF265 [39], cyclin E [40], and NIPP1 [41], have not been identified in mass spectrometric analyses of splicing complexes. The reasons for this are unclear. It may be that these proteins are less abundant in the spliceosome complex, or lack convenient tryptic sites that will facilitate detection by mass spectrometry. Thus the non-identification of a protein by mass spectrometry does not necessarily indicate its absence in the complex. The purification of the splicing complexes was done using very reliable and reproducible standard methods that have been used previously in several studies [16,28,33–37]. It is possible that hLodestar/HuF2 is incorporated into the splicing machinery through its interaction with CDC5L.

In this study, we have shown that CDC5L and hLodestar/HuF2 interact by a yeast two-hybrid assay and confirmed this interaction by co-immunoprecipitation analyses using [35 S]methionine-labeled HT1080 cell lysates and HeLa nuclear extract. In addition, our results indicate that hLodestar/HuF2 is a stable component of the human spliceosome complex. We have also shown that a truncated form of hLodestar/HuF2 (tr-hLdstr) prevented the formation of splicing complexes. The finding that tr-hLdstr blocked splicing *in vitro* indicates that this polypeptide may have a dominant-negative effect on the interaction between CDC5L and hLodestar/HuF2 in HeLa nuclear extract and suggests a possible role for hLodestar/HuF2 in pre-mRNA splicing.

How might hLodestar/HuF2 interact with a protein known to be essential for pre-mRNA splicing and still play a role in transcription termination? In recent years, evidence has emerged that the mRNA processing steps of capping, splicing, and polyadenylation occur co-transcriptionally. These processing steps influence each other's efficiency and are coordinated by transcription. Substantial evidence demonstrates an association between the RNA polymerase II (Pol II) complex and mRNA processing. Beyer and Osheim [42] used electron

microscopy of chromosome spreads to demonstrate the possibility of co-transcriptional splicing. This work was followed by evidence that splicing factors are recruited to transcriptionally active genes. It has been shown in HeLa cells that splicing factors associate with intron-containing nascent transcripts and not with intron-lacking transcripts [43].

Our study has expanded the understanding of hLodestar/HuF2's function to include a role in pre-mRNA splicing, in addition to its transcription termination function described by others. These results are an exciting addition to the SNF2 protein family's repertoire. It is possible that during the cell division cycle, hLodestar/HuF2's associates with transcription/splicing complexes in order to inhibit transcription/splicing prior to the start of mitosis and/or functions in stabilizing nuclear complexes containing splicing factors (e.g., the CDC5L complex) so that these are available for re-initiation of splicing at the end of mitosis when gene expression is re-established in cells. Further studies into hLodestar/HuF2's specific function in the pre-mRNA splicing mechanism may provide additional insights into shared mechanisms between the SNF2 protein family and co-transcriptional splicing.

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