

Multiple protein–protein interactions converging on the Prp38 protein during activation of the human spliceosome

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ABSTRACT

Spliceosomal Prp38 proteins contain a conserved amino-terminal domain, but only higher eukaryotic orthologs also harbor a carboxy-terminal RS domain, a hallmark of splicing regulatory SR proteins. We show by crystal structure analysis that the amino-terminal domain of human Prp38 is organized around three pairs of antiparallel α -helices and lacks similarities to RNA-binding domains found in canonical SR proteins. Instead, yeast two-hybrid analyses suggest that the amino-terminal domain is a versatile protein–protein interaction hub that possibly binds 12 other spliceosomal proteins, most of which are recruited at the same stage as Prp38. By quantitative, alanine surface-scanning two-hybrid screens and biochemical analyses we delineated four distinct interfaces on the Prp38 amino-terminal domain. In vitro interaction assays using recombinant proteins showed that Prp38 can bind at least two proteins simultaneously via two different interfaces. Addition of excess Prp38 amino-terminal domain to in vitro splicing assays, but not of an interaction-deficient mutant, stalled splicing at a precatalytic stage. Our results show that human Prp38 is an unusual SR protein, whose amino-terminal domain is a multi-interface protein–protein interaction platform that might organize the relative positioning of other proteins during splicing.

Keywords: pre-mRNA processing factor 38; pre-mRNA splicing; protein–protein interactions; spliceosomal B complex; spliceosome; yeast two-hybrid analysis

INTRODUCTION

Splicing entails the removal of noncoding intervening sequences (introns) from precursor messenger RNAs (pre-mRNAs) and the ligation of neighboring coding regions (exons), and it constitutes an essential step in the maturation of most eukaryotic primary protein-coding transcripts. A splicing event encompasses two consecutive transesterification reactions, steps 1 and 2, which in the vast majority of cases in humans are carried out by the major spliceosome. A hallmark of spliceosomes is their stepwise assembly from five small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4, U5, and U6 in the major spliceosome) and many non-snRNP splicing factors, which occurs de novo for each splicing event (Wahl et al. 2009; Will and Lührmann 2011). In the consensus view of constitutive splicing, a splicing process is initiated by formation of an A complex, in which U1 snRNP is bound at a 5'-splice site (SS) and U2 snRNP at a branch point sequence (BPS) of an intron. Subsequently,

the B complex is formed by incorporation of the U4, U5, and U6 snRNPs as a preformed U4/U6•U5 tri-snRNP. Neither the spliceosomal snRNPs nor the B complex contain a functional active site for the splicing transesterification reactions. Instead, the B complex must undergo major compositional and conformational rearrangements to first yield the B^{act} and, after additional rearrangements, the B* complex, which carries out the first step of splicing. Further remodeling after step 1 gives rise to the C complex, which catalyzes the second transesterification reaction, after which the mature mRNA is released as a mRNP and the remaining subunits of the spliceosome are recycled for further rounds of splicing.

Spliceosome catalytic activation involves a number of processes that depend on the activities of spliceosome-associated RNA helicases: that is, (i) removal of U1 snRNP from the 5'-SS, which requires the Prp28 protein (Chen et al. 2001); (ii)

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unwinding of U4 and U6 snRNAs, which are extensively base paired in the U4/U6•U5 tri-snRNP and in the B complex, via the Brr2 protein (Laggerbauer et al. 1998; Raghunathan and Guthrie 1998); and (iii) remodeling of the U2 snRNP-associated heteromeric splicing factors 3a and 3b around the BPS, which is facilitated by the Prp2 protein (Warkocki et al. 2009; Lardelli et al. 2010). Additional proteins have been functionally linked to spliceosome catalytic activation, including the Prp38 protein in yeast. *Saccharomyces cerevisiae* cells bearing temperature-sensitive (ts) *prp38* alleles exhibit severe splicing defects at repressive temperatures, with splicing being stalled before the first transesterification reaction (Blanton et al. 1992; Xie et al. 1998).

Prp38 is a universally conserved splicing factor and is also essential in *Drosophila melanogaster* (Andersen and Tapon 2008). Although *S. cerevisiae* (*sc*) Prp38 is a stable subunit of the U4/U6•U5 tri-snRNP, the human (*hs*) counterpart is recruited to the spliceosome independently of the tri-snRNP at the B-complex stage, together with a group of eight other non-snRNP proteins (*hs*Snu23, *hs*MFAP1, *hs*Snu1, *hs*RED, *hs*FBP21, *hs*UBL5, *hs*NPW38, and *hs*NPW38BP) (Agafonov et al. 2011). *hs*Prp38 and the latter group of non-snRNP proteins are released again during the next step of spliceosome maturation, the formation of the activated B^{act} complex (Agafonov et al. 2011). Thus, we refer to *hs*Prp38 and the other proteins recruited and released at the same stages as “B-specific” factors.

Although an amino-terminal domain (NTD) of unknown structure and function is found in all Prp38 orthologs, only higher eukaryotic Prp38 proteins additionally contain a carboxy-terminal arginine–serine (RS) repeat-containing region (Fig. 1A–C). RS domains are a signature of serine/arginine-rich splicing regulatory proteins (SR proteins) (Long and Cáceres 2009). The RS domains of SR proteins can be serine-phosphorylated by SR protein kinases (SRPKs) and can interact with other SR proteins and pre-mRNAs in a phosphorylation-dependent manner (Long and Cáceres 2009). Consistent with the carboxy-terminal region of *hs*Prp38 constituting an

authentic RS domain, it has been found to bind other SR proteins and SRPKs in a yeast two-hybrid (Y2H) assay (Hegele et al. 2012). In canonical SR proteins, the RS domain is preceded or followed by one or several RNA recognition motif (RRM) and/or Zinc-finger (ZnF) domains, which often

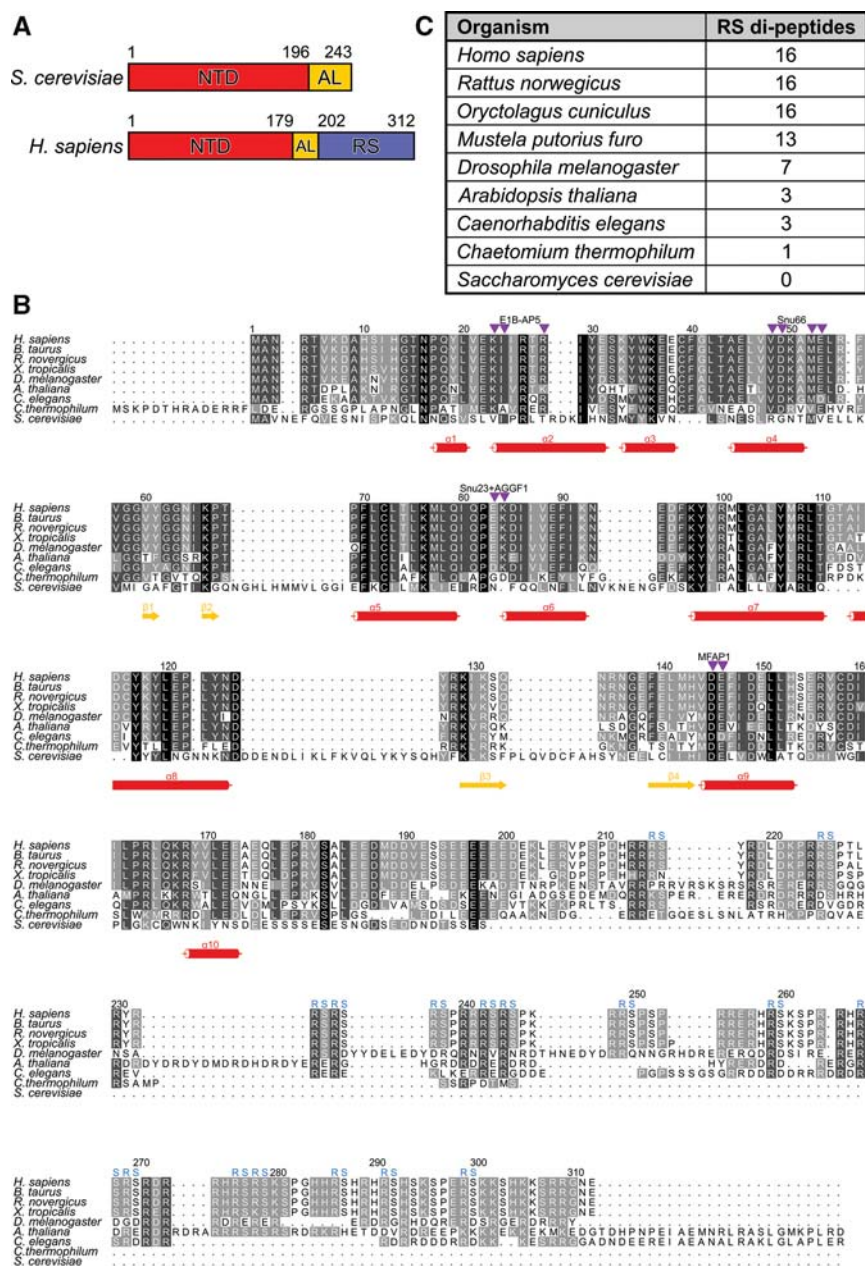


FIGURE 1. Domain organization and conservation of Prp38 orthologs. (A) Domain organization of *Saccharomyces cerevisiae* and *Homo sapiens* Prp38 proteins. Numbers indicate domain borders. (B) Multiple sequence alignment of Prp38 orthologs. Numbers above the alignment refer to the human protein. Secondary structure elements based on the crystal structures of *hs*Prp38^{NTD} and *hs*Prp38^{NTD+} are indicated by red cylinders (α-helices) and yellow arrows (β-strands). Residues, at which substitutions selectively interfere with binding to specific *hs*Prp38-interacting proteins, according to clusters IV–VII (see Fig. 4) are pointed out by purple triangles. Blue “RS” symbols above the alignment indicate RS dipeptides found in the carboxy-terminal region of *hs*Prp38. (C) Number of RS repeats in Prp38 orthologs of different species. A higher number of RS repeats correlates with a higher complexity of the host.

bind exonic splicing enhancer (ESE) sequences on pre-mRNAs (Long and Cáceres 2009). More rarely, SR proteins contain other RNA-binding domains, such as RNA helicase or PWI domains, in addition to or instead of RRM or ZnFs (Long and Cáceres 2009). There are only very few cases of SR proteins with RNA-unrelated additional domains, such as a cyclophilin domain in the case of CypRS (Stegmann et al. 2009) or a kinase domain in ClkSty-1 (Long and Cáceres 2009). The Prp38 NTD is unrelated to known RS domain-associated domains in other SR proteins. Presently, it is unclear which precise functions are exerted by the universal Prp38 NTD and which additional, higher eukaryote-specific functions are maintained through the RS domain.

Here, we elucidated the crystal structure of the *hsPrp38* NTD (*hsPrp38*^{NTD}) and showed by Y2H analysis that it binds a large number of other spliceosomal proteins, including, but not limited to, previously characterized Prp38 interactors. We also delineated four distinct binding sites for several interacting proteins on the *hsPrp38* NTD and found that at least two Y2H interaction partners can bind the NTD simultaneously in vitro. When added in excess to splicing reactions, the isolated NTD stalls the spliceosome at a pre-catalytic B-complex stage. Our results show that *hsPrp38* is an unusual SR protein, in which the RS domain is coupled to a multi-interface protein-protein interaction (PPI) domain.

RESULTS

Crystal structure analysis suggests that *hPrp38*^{NTD} is a protein-protein interaction domain

Humans bear two *scPrp38*-like proteins, produced from separate genes, *hsPrp38A* (UniProt ID: Q8NAV1; Gene ID: 84950) and *hsPrp38B* (UniProt ID: Q5VTL8; Gene ID: 55119). The two proteins show 38.5% sequence identity for 112 aligned residues, but *hsPrp38B* (546 residues) is significantly longer than *hsPrp38A* (312 residues) and bears a number of insertions and a carboxy-terminal appendix (not shown). Both proteins are ubiquitously produced in human tissues (<http://www.proteinatlas.org>) but so far only *hsPrp38A* has been found in spliceosomal complexes (Wahl et al. 2009; Agafonov et al. 2011).

hsPrp38 contains an NTD (residues 1–179) of unknown fold and function, which is universally conserved in Prp38 orthologs, followed by a stretch rich in acidic amino acids (acidic linker, AL; residues 180–202) and a carboxy-terminal region that harbors a series of RS dipeptide repeats (residues 203–312), resembling the RS domains of SR proteins (Fig. 1A,B). Although it is lacking from *scPrp38*, an equivalent of the RS domain is also found in other higher eukaryotic Prp38 orthologs, and the number of RS repeats in this region increases from simple multicellular organisms (three RS repeats in *Caenorhabditis elegans*) via more complex invertebrates (seven RS repeats in *Drosophila melanogaster*) to mammalian Prp38 proteins (16 RS repeats in *hsPrp38*; Fig. 1B,C).

The conserved Prp38 NTD does not bear obvious sequence similarity to known RNA-binding domains as found in canonical SR proteins. To start investigating its possible functions, we determined a crystal structure of *hsPrp38*^{NTD} (residues 1–179) at 1.28-Å resolution via single-wavelength anomalous dispersion (SAD), using selenomethionine-derivatized protein (Table 1). Crystals of *hsPrp38*^{NTD} contained a single molecule per asymmetric unit, all residues of which were well defined in the electron density. The core of the *hsPrp38*^{NTD} structure is formed by three pairs of antiparallel α -helices ($\alpha 4/\alpha 5$, $\alpha 6/\alpha 7$, and $\alpha 8/\alpha 9$), which are connected and surrounded by long loops, β -hairpins, and four additional helices ($\alpha 1$, $\alpha 2$, and $\alpha 3$ on one end and $\alpha 10$ on the other; Fig. 2A). We also determined a structure of *hsPrp38*^{NTD+} (residues 1–205), containing NTD and AL, at 1.9 Å resolution by molecular replacement, using the *hsPrp38*^{NTD} structure as a search model (Table 1). Again, the first 179 residues were clearly visible in the electron density, whereas residues 180–205, including the AL, could not be traced, presumably because of intrinsic disorder. The structure of *hsPrp38*^{NTD+} is very similar to that of *hsPrp38*^{NTD} (root-mean-square deviation [rmsd] of 0.54 Å for 165 structurally equivalent Ca atoms) with the exception of the last 14 ordered residues, 166–179. In *hsPrp38*^{NTD+} this region forms a loop that folds back onto the tandem helical repeats $\alpha 6/\alpha 7$ and $\alpha 8/\alpha 9$ instead of forming a short α -helix ($\alpha 10$) as seen in *hsPrp38*^{NTD} (Fig. 2A,B).

As expected from sequence comparisons, the structure of *hsPrp38*^{NTD} lacks similarity to known RNA-binding domains. Instead, a similarity search with the Dali server (Holm and Sander 1998) revealed that the central helical repeats of *hsPrp38*^{NTD} structurally resemble HEAT repeats as found, for example, in the phosphatase 2A subunit PR65 (root-mean-square deviation of 2.9 Å for 105 structurally equivalent Ca atoms to PDB ID 1B3U; Groves et al. 1999). As HEAT repeat regions comprise versatile PPI platforms (Palidwor et al. 2009), we next investigated the possibility that the NTD of Prp38 proteins functions as a PPI element.

The spliceosomal *hsPrp38*^{NTD} interactome

A recent Y2H analysis identified PPIs among approximately 240 human spliceosomal proteins (Hegele et al. 2012). This screen included the AL and carboxy-terminal RS domain of *hsPrp38*, whereas the universally conserved NTD was not represented in this study. Therefore, to test if *hsPrp38*^{NTD} acts as a protein-binding platform in the spliceosome, we performed Y2H-based interaction screens with *hsPrp38*^{NTD} or full-length *hsPrp38* (*hsPrp38*^{FL}) as bait and the above prey collection of human spliceosomal proteins represented by more than 440 clones. Bait and prey constructs were tested in a pairwise manner with double (*hsPrp38*^{FL}) or fourfold (*hsPrp38*^{NTD}) redundancy. Prey constructs that yielded colonies in both independent tests (in the case of *hsPrp38*^{FL}) or in at least three independent experiments (in the case of

TABLE 1. Crystallographic data

Data collection	<i>hsPrp38</i> ^{NTD}	<i>hsPrp38</i> ^{NTD} (SeMet)	<i>hsPrp38</i> ^{NTD+}
Wavelength [Å]	0.918	0.980	0.895
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell			
<i>a</i> [Å]	41.9	41.9	42.6
<i>b</i> [Å]	61.9	61.1	59.4
<i>c</i> [Å]	67.7	69.0	68.8
Resolution [Å] ^a	19.0–1.28 (1.33–1.28)	35.0–1.71 (1.75–1.71)	45.0–1.90 (1.95–1.90)
Unique reflections	46138 (4542)	36884 (2768)	14297 (1027)
Completeness	99.9 (100.0)	99.9 (99.9)	99.7 (98.4)
<i>I</i> / σ (<i>I</i>)	37.6 (2.9)	13.4 (2.1)	13.0 (1.7)
Redundancy	7.2 (7.2)	3.2 (3.1)	4.5 (4.5)
<i>R</i> _{Sym} ^b	0.055 (0.734)	0.057 (0.552)	0.087 (0.867)
Phasing			
No. of sites		5	
FOM ^c		0.68	
Refinement			
Reflections test set	2318		716
Final model			
Protein residues	179		179
Protein atoms	1733		1518
Water oxygens	247		98
<i>R</i> _{work} ^d	0.1264 (0.1894)		0.1575 (0.2033)
<i>R</i> _{free} ^d	0.1502 (0.2188)		0.2078 (0.2596)
Aver. B-factor [Å ²]	20.1		32.2
Rmsd ^e			
Bond lengths [Å]	0.011		0.06
Bond angles [°]	1.35		0.99
Ramachandran ^f			
Favored [%]	98.6		97.8
Allowed [%]	0.9		2.2
Outliers [%]	0.5		0
PDB entry	4RZ9		4RZA

^aValues for the highest resolution shell in parentheses.

^b $R_{Sym} = \sum_h [n(n-1)]^{1/2} \sum_i |I_{h,i} - I_{h,i}| / \sum_i \sum_j |I_{h,i} - I_{h,j}|$, where I_h is the mean intensity of symmetry-equivalent reflections and n is the redundancy.

^cFOM = figure of merit = $m = 1/2 F(hkl)_{best} / 1/2 F(hkl)_{1/2}$, in which $F(hkl)_{best} = \sum_{\alpha} |P(\alpha) F_{hk}(\alpha)| / \sum_{\alpha} P(\alpha)$, P is the phasing power, and α is the phase angle.

^d $R = \sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$; $R_{work} - hkl \notin T$; $R_{free} - hkl \in T$; where T is the test set.

^eRmsd, root-mean-square deviation.

^fCalculated with MolProbity (<http://molprobity.biochem.duke.edu/>).

hsPrp38^{NTD}) were considered potential binding partners (Fig. 3A, top and middle). We screened in parallel specific domain constructs of other spliceosomal proteins, which yielded very different interaction candidates (Fig. 3A, bottom), demonstrating the specificity of the Y2H results.

Based on these criteria, 12 putative, direct interaction partners of *hsPrp38*^{NTD} were identified (Fig. 3A,B). One major group of Y2H interactors consists of other B-specific proteins (i.e., *hsSnu23*, *hsMFAP1*, and *hsRED*). *hsPrp38*^{NTD} also showed interactions with the tri-snRNP proteins, *hsPrp31* and *hsSnu66*, which are also recruited at the B-complex stage, the Prp19-related protein *hsSKIP*, which starts to get recruited at B-complex stage but is fully present only at the following B^{act} stage, as well as with *hsNY-CO-10/hsCwc27*, which first enters

at the B^{act} stage (Agafonov et al. 2011). In addition, there were *hsPrp38*^{NTD} Y2H interactions with several splicing factors that are recruited early during spliceosome assembly (Agafonov et al. 2011) (i.e., *hsRBM5*, *hsRBM10*, *hsSF3b125*, *hsE1B-AP5*, and *hsAGGF1*). *hsRBM5*, *hsRBM10*, and *hsSF3b125* bind directly to the spliceosomal discard and disassembly factor *hsPrp43* (which is at least in part recruited during A complex formation; Agafonov et al. 2011), to the spliceosomal activation factor *hsPrp19*, and to the alternative splicing factor *hsSR140*, respectively (Hegele et al. 2012). *hsE1B-AP5* interacts with heterogeneous nuclear (hn) RNP proteins and, like *hsAGGF1*, with the U1 snRNP component *hsU1C* (Hegele et al. 2012). Notably, *hsPrp38*^{NTD} did not interact with any of the canonical SR proteins, SRPKs, or any

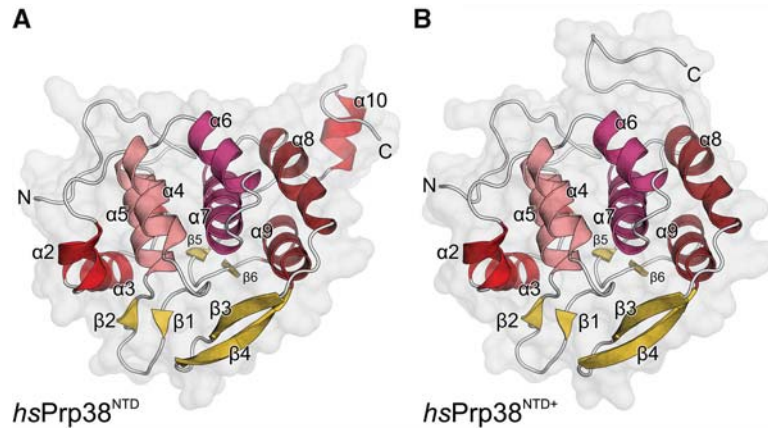


FIGURE 2. Crystal structures of *hsPrp38* amino-terminal domains. Cartoon representation of (A) the crystal structure of *hsPrp38*^{NTD} (residues 1–179) and (B) the crystal structure of *hsPrp38*^{NTD+} (residues 1–205). α -Helices—red colors; β -strands—yellow; loops—gray. Three α -helical repeats forming the cores of the structures ($\alpha 4/\alpha 5$, $\alpha 6/\alpha 7$, and $\alpha 8/\alpha 9$) are shown in different shades of red.

proteins, besides *hsSKIP* and *hsNY-CO-10/hsCwc27*, that are recruited after the B-complex stage (Agafonov et al. 2011).

As expected, *hsPrp38*^{FL} interacted with proteins previously identified as interactors of its AL/RS domain (*hshRNPS1*, *hsSRSF4*, and *hsSRPK2*; Hegele et al. 2012), and with a subset of the *hsPrp38*^{NTD} binding partners (*hsMFAP1* and *hsSnu66*). It showed additional interactions not observed with *hsPrp38*^{NTD} or the AL/RS domain (*hsSRSF2*, *hsLuc7*, *hsPrp16*, *hsNKAP*, and *hsCGI-79*; Fig. 3A,B). These observations could indicate that the presence of the AL/RS domain can influence protein binding at the NTD and vice versa, as well as that some *hsPrp38* interactors might recognize epitopes on both the NTD and the RS domain. However, differences in the interaction profiles might also reflect differences in the Y2H constructs (such as steric constraints due to the proximity of the fused DNA-binding domain) and/or differences in the expression levels for the various constructs. Therefore, we presently consider all proteins that show up as Y2H interactors of any of the tested *hsPrp38* regions as candidate binding partners. Together, our Y2H results suggest that the NTD of *hsPrp38* serves as a PPI hub in the course of assembly of the spliceosomal B complex.

Systematic mutational analysis identifies four distinct protein interaction interfaces on *hsPrp38*^{NTD}

Several of the *hsPrp38*^{NTD}/*hsPrp38*^{FL} interaction partners identified here are simultaneously present in a spliceosomal complex, such as the B-specific proteins (Agafonov et al. 2011). Thus the question arises: Do these proteins bind to the same or overlapping sites on the *hsPrp38* surface, or do certain groups of interaction partners recognize distinct binding sites enabling simultaneous binding of two or more proteins. Based on hydrophobicity, charge distribution, or conservation patterns, no obvious protein interaction

interfaces were directly apparent from the *hsPrp38*^{NTD} or *hsPrp38*^{NTD+} structures. Therefore, we systematically substituted surface-exposed amino acids, individually or in neighboring pairs, which could potentially contribute to protein binding, with alanines, and we tested interactions of the resulting 56 *hsPrp38*^{NTD} variants with the previously identified interactors of wild-type (wt) *hsPrp38*^{NTD} in a Y2H array. In this approach, all candidates were tested in triplicates and in parallel against each alanine mutant version of *hsPrp38*^{NTD}. In principle, specific Y2H pairs might result in cell toxicity and thus give rise to weaker growth. However, during selection for the diploid strains after mating, both baits and preys are constitutively expressed.

In this step, we obtained uniform growth across the entire array, demonstrating a lack of toxicity for any bait/prey combination. Because our Y2H system is tuned toward very low protein production levels to increase specificity, typically no toxic effects are observed (Worbeck et al. 2012). The setup, therefore, enabled us to reliably quantify the growth as a measure of relative interaction signal (pixel intensities of grayscale bitmap images).

We normalized the growth signals within each array to the *hsPrp38*^{NTD}–*hsMFAP1* interaction, as this interaction gave rise to the most robust and strongest growth signal among all *hsPrp38*^{NTD} interactions and was invariant in all but one mutant pairing. Overall, highly correlated values were obtained for interacting pairs, suggesting that growth is not affected by possible differences in the expression levels of bait and prey constructs. Mean values of triplicate experiments for *hsPrp38*^{NTD} variants were then hierarchically clustered revealing patterns of decreased or increased interaction with the 12 previously identified wt *hsPrp38*^{NTD} interaction partners (Fig. 4A,B).

More than half of the mutant constructs (31) gave rise to interaction patterns very similar to wt *hsPrp38*^{NTD} (cluster I), suggesting that no critical protein–protein contact residues were affected by the corresponding residue exchanges. All other variants led to reduced Y2H interactions or, in a single case, to an enhanced Y2H interaction. Based on pattern similarity in the cluster analysis, we grouped these variants into six distinct sets (clusters II–VII; Fig. 4A,B). *hsPrp38*^{NTD} mutations in clusters II and III abolished all interactions except with *hsMFAP1* or with *hsMFAP1* and *hsE1B-AP5*, respectively. Residues exchanged by the mutations of clusters II and III are scattered all over the surface of *hsPrp38*^{NTD}, and thus do not point to a confined interaction region on wt *hsPrp38*^{NTD}. Rather it suggests that this set of amino acid changes affects protein expression, folding, or stability. In contrast, clusters IV–VII represent *hsPrp38*^{NTD}

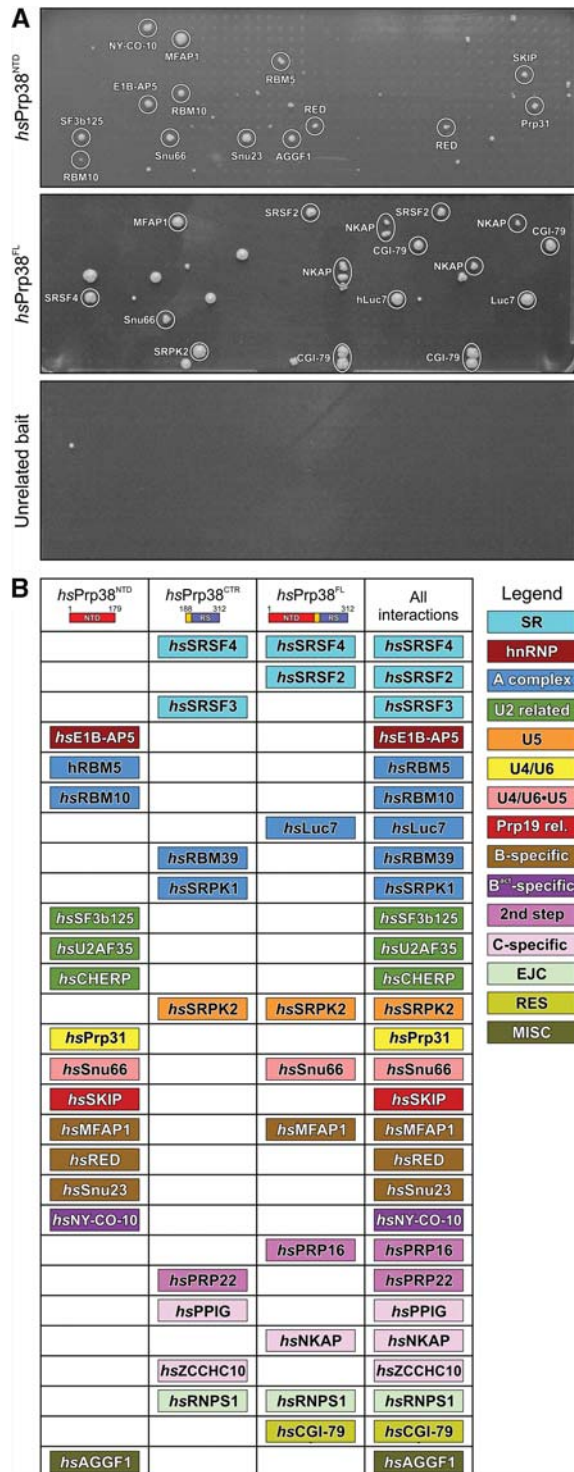


FIGURE 3. *hsPrp38* binding partners identified by yeast two-hybrid analyses. (A) Representative Y2H screens of *hsPrp38*^{NTD} (top), *hsPrp38*^{FL} (middle), and an unrelated bait as negative control (bottom). Y2H hits were considered putative interactions if they appeared in at least 75% of the repetitions. (B) Interaction partners of *hsPrp38*^{NTD} (this study), a carboxy-terminal region (CTR) of *hsPrp38* (comprising the AL and RS domain; residues 188–312; Hegele et al. 2012) and *hsPrp38*^{FL} (this study) are listed and color-coded according to their affiliation with spliceosomal subcomplexes or groups of proteins (legend on the right).

variants, for which only a selected subset of Y2H interactions was changed, whereas Y2H interactions with all other *hsPrp38*^{NTD} Y2H interactors were hardly affected. The exchanged residues in clusters IV–VII show distinct local distributions on the *hsPrp38*^{NTD} surface (Fig. 4C). The large set of mutants that show differential Y2H interactions provide further evidence for the specificity of our assays.

In cluster IV, mutations K23A/I24A and R28A are located at the amino terminus and center of helix α_2 , respectively, and interfered specifically with *hsE1B-AP5* binding, whereas all other *hsPrp38*^{NTD} interaction partners showed wt-like growth signals. Thus, *hsE1B-AP5* might directly interact with the helix α_2 region of *hsPrp38*^{NTD} (Fig. 4C). In cluster V, three double mutations selectively altered *hsSnu66* binding. Of these, V48A/D49A and M52A/E53A are located in *hsPrp38*^{NTD} helix α_4 , where V48 is engaged in hydrophobic interactions with F90 of helix α_6 and V99 of helix α_7 , whereas M52 interacts with V99 and engages in a hydrogen bond with D49 of helix α_4 . The corresponding mutations might thus not only change the local physico-chemical surface properties, but the absence of the intramolecular contacts involving the affected residues could additionally influence positioning of helix α_4 . The E45A/L46A mutations at the amino terminus of helix α_4 increased *hsSnu66* binding relative to wt *hsPrp38*^{NTD}. Although E45 is a surface-exposed residue, L46 binds to L42 located in the loop preceding helix α_4 , which could again change the position of this secondary structure element. These results suggest that helix α_4 and the immediate surroundings on *hsPrp38*^{NTD} represent a binding site for *hsSnu66* (Fig. 4C). Construct M52A/E53A, in addition to *hsSnu66*, also decreased Y2H interactions with *hsRBM5*, *hsRBM10*, and *hsSF3b125*, suggesting that these proteins take advantage of an interaction region on *hsPrp38*^{NTD}, which at least partly overlaps with the *hsSnu66*-interaction site (Fig. 4C). Residues affected by the E83A and E83A/K84A exchanges (cluster VI) are positioned in a rather flat surface area formed by the loop connecting helix α_5 to helix α_6 . The corresponding mutations abolished Y2H interactions of *hsPrp38*^{NTD} with *hsAGGF1* and *hsSnu23*, suggesting that *hsAGGF1* and *hsSnu23* share an interaction site in the corresponding region (Fig. 4C). Finally, residues affected by the D145A/E146A exchanges of cluster VII are located at the amino-terminal tip of helix α_9 and the residue exchanges selectively decreased the *hsMFAP1* and *hsSF3b125* interactions (Fig. 4C).

hsRED, *hsPrp31*, *hsSKIP*, and *hsNY-CO-10*/*hsCwc27* interaction sites could not be localized unambiguously with the set of *hsPrp38*^{NTD} mutant constructs used. Prey constructs representing these four proteins already showed weaker growth with the wt *hsPrp38*^{NTD} bait than the other identified interactors, making it more difficult to reliably detect effects of single *hsPrp38*^{NTD} point mutations on these interactions. In addition, these proteins may involve more extensive contact surfaces than those tested in the individual constructs or may interact at sites not well covered in our alanine scan.

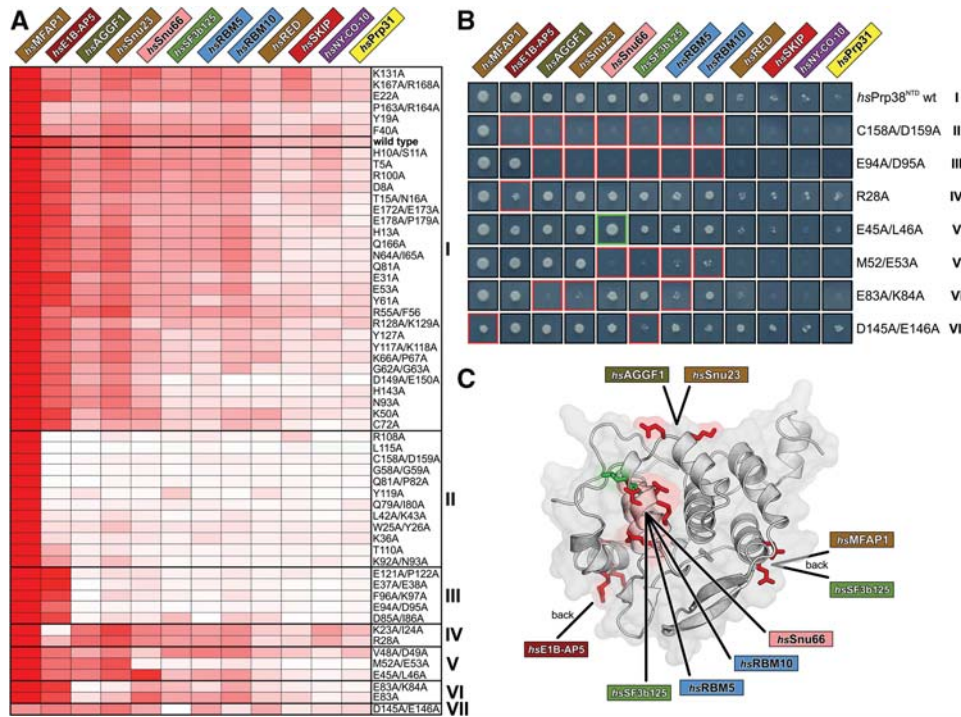


FIGURE 4. Binding site mapping. (A) Fifty-six single or double alanine variants of *hsPrp38*^{NTD} are clustered according to the degree of similarity in their effects on the interactions with the 12 identified *hsPrp38*^{NTD} binding partners (clusters I–VII on the right). As a measure of relative binding strength, the growth signal of Y2H hits was quantified, normalized to the growth signal of the *hsPrp38*^{NTD}–*hsMFAP1* interaction and presented in a heat map by a white-to-red color gradient. White boxes—no growth; red boxes—strong growth. The wt binding profile is boxed in cluster I. (B) Growth of representative Y2H colonies of the 12 *hsPrp38*^{NTD} binding partners with respective *hsPrp38*^{NTD} variants of all seven clusters on selective agar (I–VII). Red or green boxes indicate reduction or increase of the growth signal relative to wt *hsPrp38*^{NTD}, respectively. V↑—subgroup of cluster V with increased interaction to *hsSnu66*; V↓—subgroup of cluster V with reduced interaction to *hsSnu66*. (C) Cartoon representation of *hsPrp38*^{NTD} with clusters IV–VII mapped to the protein surface. Residues mutated in clusters IV–VII that negatively affect binding of an interaction partner are colored in red; the residue that upon mutation positively affects binding of *hsSnu66* is colored in green.

Taken together, our growth signal–based, quantitative Y2H analysis provided amino acid–level information to distinguish four distinct interaction interfaces distributed across various surfaces of the compactly folded *hsPrp38*^{NTD}: Relative to the *hsSnu66*-binding area in the center of one face of *hsPrp38*^{NTD} (which is possibly also used by *hsRBM5*, *hsRBM10*, and *hsSF3b125*), a *hsSnu23*/*hsAGGF1*-binding site lies at the top on the same face, whereas a *hsE1B-AP5* interaction site and a *hsMFAP1*/*hsSF3b125*-binding site are located on the right and left side, respectively, of the opposite face of *hsPrp38*^{NTD} (Fig. 4C). Although proteins mapping to the same or overlapping interfaces on *hsPrp38*^{NTD} (such as *hsSnu66*/*hsRBM5*/*hsRBM10*/*hsSF3b125*, *hsSnu23*/*hsAGGF1*, and *hsMFAP1*/*hsSF3b125*) might thus bind mutually exclusively to *hsPrp38*, proteins that take advantage of spatially separated contact regions on *hsPrp38*^{NTD} have the potential to bind concomitantly to the protein.

Concomitant binding of several Y2H interactors to Prp38^{NTD}

To validate some of the Y2H interactors and their putative binding sites on *hsPrp38*^{NTD} and to confirm that two

proteins with spatially separated, putative binding sites can interact concomitantly with Prp38^{NTD}, we performed biochemical binding assays. We recombinantly produced and purified *hsMFAP1* (cluster VII) and *hsSnu23* (cluster VI) and analyzed complex formation of these proteins with *hsPrp38*^{NTD} by analytical size exclusion chromatography (SEC). Instead of full-length *hsMFAP1*, we used a long, soluble fragment of the protein (residues 30–344, identified by amino-terminal sequencing and mass spectrometric fingerprinting), which resulted from endogenous limited proteolysis upon recombinant production of full-length *hsMFAP1* in *Escherichia coli*. Consistent with the strong and reproducible interaction of *hsPrp38*^{NTD} and *hsMFAP1* in the Y2H screens, *hsPrp38*^{NTD} and *hsMFAP1*^{30–344} coeluted from the SEC column at a smaller volume compared with the individual proteins (Fig. 5A, panels I, III, IV). In contrast, *hsPrp38*^{NTD} variants bearing the D145A/E146A double exchanges, which led to abrogation of the *hsMFAP1* Y2H interaction, or only the D145A single exchange did not coelute with *hsMFAP1*^{30–344} (Fig. 5A, panels II, V, VI). These results suggest that our Y2H analyses indeed represent direct, binary *hsPrp38*^{NTD} interactions and that our systematic mutational analyses point to

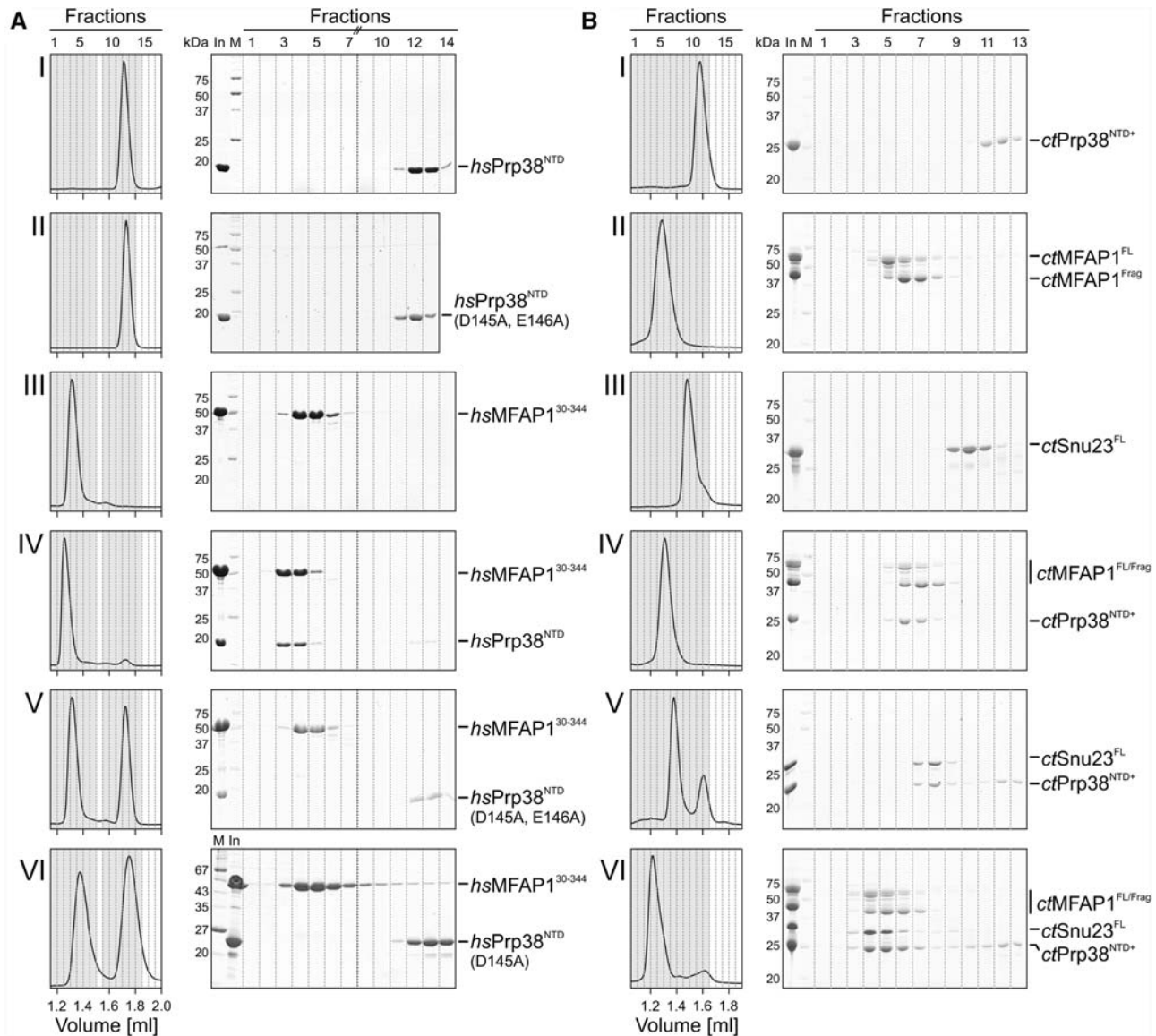


FIGURE 5. Analysis of Prp38-MFAP1, Prp38-Snu23, and Prp38-MFAP1-Snu23 complexes. (A) SEC elution profiles and corresponding SDS-PAGE gels of wt *hsPrp38*^{NTD} or cluster VII mutation (D145A/E146A, D145A) with a large soluble fragment of *hsMFAP1* (residues 30–344). Fractions with a gray background are analyzed on the SDS gels. (B) Binary (panels I–V) and ternary (panel VI) SEC interaction analysis of wt *ctPrp38*^{NTD+} (residues 1–220) with *ctMFAP1* and *ctSnu23*. *ctMFAP1*^{Frag}—fragment obtained by endogenous limited proteolysis during production and purification of *ctMFAP1*^{FL}. Fractions with a darker gray background were analyzed on the SDS gels.

direct contact sites on *hsPrp38*^{NTD} with the respective interactor.

In contrast to *hsMFAP1*³⁰⁻³⁴⁴, *hsSnu23* did not stably interact with *hsPrp38*^{NTD} (or *hsPrp38*^{NTD+}) alone or with *hsPrp38*^{NTD} (or *hsPrp38*^{NTD+}) prebound to *hsMFAP1*³⁰⁻³⁴⁴ in SEC. Under the nonequilibrium SEC conditions, only kinetically and thermodynamically relatively stable complexes are expected to persist. We reasoned that the corresponding interactions might be more stable at ambient temperatures for proteins originating from a thermotolerant species. We therefore recombinantly produced Prp38^{NTD+}, MFAP1, and Snu23 from the thermotolerant fungus, *Chaetomium*

thermophilum (*ct*), and repeated the SEC analysis. Our *ctMFAP1* preparation contained both the full-length protein *ctMFAP1*^{FL} and a long fragment *ctMFAP1*^{Frag}, which appeared during recombinant production and was copurified. Indeed, *ctPrp38*^{NTD+} (residues 1–220 of *ctPrp38*) stably interacted with *ctMFAP1*^{FL/Frag}, as well as with *ctSnu23*, in SEC (Fig. 5B, panels I–V; note that isolated *ctMFAP1*^{FL/Frag} and their complexes with *ctPrp38*^{NTD+} eluted in the same fractions, possibly because of an unusual shape of *ctMFAP1*^{FL/Frag}, but that binding is clearly revealed by shifting of *ctPrp38*^{NTD+} into the *ctMFAP1*^{FL/Frag} elution fractions). To test whether both *ctMFAP1*^{FL/Frag} and *ctSnu23*

bind to *ctPrp38*^{NTD+} simultaneously, we mixed all three proteins and performed SEC runs. The three proteins (i.e., ternary complexes containing *ctPrp38*^{NTD+}, *ctSnu23*, and either *ctMFAP1*^{FL} or *ctMFAP1*^{frag}) coeluted at a smaller volume than any of the binary combinations (Fig. 5B, panel VI). Although formation of the ternary complex may be further supported by direct *ctMFAP1*–*ctSnu23* interactions, the above results together suggest that Prp38 can indeed utilize different interfaces to bind multiple proteins at the same time and thus behaves as a true multi-interface protein interaction platform.

An excess of *hsPrp38*^{NTD+} stalls splicing after B-complex formation

As only the NTD and AL are universally conserved among Prp38 orthologs, we next tested whether this region alone is sufficient to mediate spliceosome association of *hsPrp38* and to support splicing. To this end, we added increasing amounts of recombinantly produced *hsPrp38*^{NTD+} (residues 1–205) to splicing-competent HeLa nuclear extract and monitored the effects on splicing of MINX pre-mRNA and on spliceosome assembly. We observed increasing inhibition of splicing at increasing concentrations of *hsPrp38*^{NTD+} compared with the control reaction lacking *hsPrp38*^{NTD+} (Fig. 6A, cf. lanes 6–9 to lane 5), with complete inhibition of both steps of splicing at 36 μ M *hsPrp38*^{NTD+} or above. Monitoring splicing complex formation on native gels indicated that spliceosome assembly was stalled after B-complex formation and does not proceed to activation and catalysis (Fig. 6B, cf. lanes 7–10 to lane 6), consistent with a role for full-length *hsPrp38* in the subsequent activation step. In contrast, when we used *hsPrp38*^{NTD+,D145A} (bearing a residue exchange that led to abrogation of its interaction with *hsMFAP1*^{30–344}; Fig. 5A, panel VI) instead of wt *hsPrp38*^{NTD+} in the assay, only very mild inhibition of splicing was observed (Fig. 6A, cf. lanes 10–13 to lane 5) and spliceosome assembly readily proceeded to the C-complex stage (Fig. 6B, compare lanes 11–14 to lane 6), suggesting that interaction of *hsPrp38*^{NTD+} with MFAP1 is important for its splicing inhibition activity. These results indicate that *hsPrp38*^{NTD+} is recruited to spliceosomes at the B-complex stage and interferes with spliceosome catalytic activation by replacing the endogenous protein or by competing with the endogenous protein for some of its functional interactions. Alternatively, *hsPrp38*^{NTD+} might sequester other splicing factors in an inactive complex, possibly through *hsMFAP1* association, before interaction with the spliceosome.

DISCUSSION

In contrast to the snRNPs and their stably associated factors, comparatively little is presently known about the functions of non-snRNP proteins in the spliceosome. In humans, a group of nine non-snRNP, B-specific proteins joins the spliceosome during B-complex formation and leaves again during the B-to-B^{act} transition (Agafonov et al. 2011). Here, we have structurally and functionally dissected the B-specific protein, *hsPrp38*. *hsPrp38* exhibits a domain organization reminiscent of SR proteins, with a universally conserved NTD that is connected to a higher eukaryote-specific, carboxy-terminal RS domain via a short AL. We find that the NTD mediates a multitude of intraspliceosomal PPIs, several of which might have to be realized concomitantly to drive spliceosomes into catalytic activation.

hsPrp38 is an unconventional SR protein

Although canonical SR proteins contain an RS domain with one or more RNA-binding domains (Long and Caceres 2009), our crystal structure analyses show that the RS domain of *hsPrp38* is associated with an α -helical repeat-containing NTD, reminiscent of PPI domains. Indeed, using a comprehensive Y2H approach, we find that 12 spliceosomal proteins show Y2H interactions with the *hsPrp38* NTD. Our screen not only uncovered proteins that have previously been reported to bind Prp38 orthologs in other organisms (i.e., MFAP1 [Andersen and Tapon 2008] or Snu23 [Krogan et al. 2006; Pandit et al. 2009]), but also revealed many additional putative *hsPrp38*^{NTD} binding partners. In principle, some of the Y2H interactions may be indirect and mediated by endogenous components of the yeast splicing apparatus, as yeast and mammalian splicing factors are

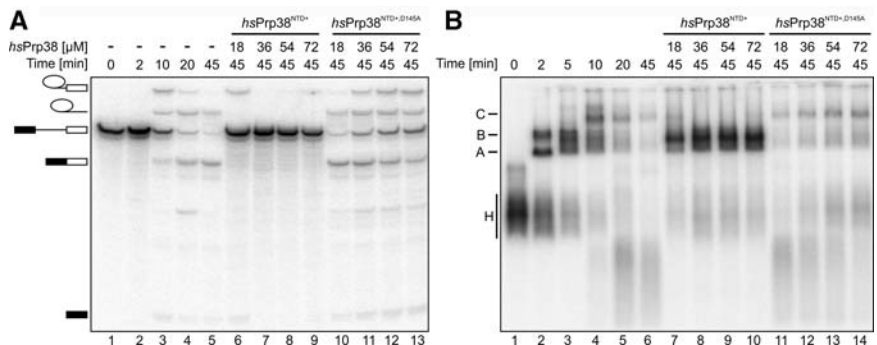


FIGURE 6. Dominant-negative effects of *hsPrp38*^{NTD+} on splicing. (A) In vitro splicing of MINX pre-mRNA monitored on a denaturing gel. Educts, intermediates, and products of the reactions are identified on the left. Lanes 1–5—time course of splicing (time points indicated above the gel) in the absence of *hsPrp38*^{NTD+}. Lanes 6–9—effects of adding increasing amounts of *hsPrp38*^{NTD+} (lanes 6–9) or *hsPrp38*^{NTD+,D145A} (lanes 10–13; indicated above the gel) to the reaction. (B) Native gel monitoring the kinetics of spliceosome assembly (time points above the gel) in the absence (lanes 1–6) or presence of increasing amounts of *hsPrp38*^{NTD+} (lanes 7–10) or *hsPrp38*^{NTD+,D145A} (lanes 11–14; indicated above the gel). Emerging complexes are identified on the left; H—hnRNP complexes; A, B, C—spliceosomal A, B, and C complexes.

generally well conserved. However, our observation of Y2H interactions between *hsPrp38*^{NTD} and several human factors that are not present in yeast (*hsMFAP1*, *hsRED*, *hsE1B-AP5*, *hsAGGF1*, *hsRBM5*, *hsRBM10*) argues against this possibility. As two tested Y2H interactions (with *MFAP1* and *Snu23*) prevailed in SEC analyses, the detected Y2H interactions likely correspond to direct, binary interactions in the spliceosome. By using proteins from two different organisms, our results also suggest that these *hsPrp38*^{NTD} interactions are evolutionarily conserved. The observed differences in *Prp38*^{NTD}-*Snu23* stability between human and *C. thermophilum* could indicate species-specific variations in binding affinities, at least under the buffer/temperature conditions used in the present experiments.

One strength of the Y2H system is to detect weak and transient interactions (Vinayagam et al. 2009; Worseck et al. 2012), which may contribute significantly to the compositional and conformational dynamics during spliceosome assembly. Therefore, rather than dismissing interactions that cannot be recapitulated in more stringent biochemical or biophysical assays, the different interaction assays should be considered complementary (Stelzl 2014). In any case, our results define *hsPrp38* as an unusual SR protein, in which a carboxy-terminal RS domain is associated with an amino-terminal PPI domain.

***hsPrp38* is a major PPI hub in the spliceosome**

High-throughput approaches, such as affinity purification coupled to mass spectrometry and Y2H assays, have been used to map PPIs in multiprotein complexes (Hegele et al. 2012), specific cellular processes (Weimann et al. 2013), or even entire proteomes (Woodsmith and Stelzl 2014). The deduced interaction networks show that the majority of the involved proteins interact with one or a few partners, whereas a limited set of hub proteins exhibit many interactions (Barabási and Oltvai 2004). Hub proteins can be further classified as “date” hubs, which contact their various interaction partners one at a time, and “party” hubs, which interact with many or all of their partners simultaneously, giving rise to multisubunit complexes (Han et al. 2004; Gursoy et al. 2008). Although date hubs typically exhibit a single interface or overlapping interfaces for the sequential interaction with their partners, party hubs are multi-interface proteins with distinct interaction surfaces for their interactors (Kim et al. 2006). In addition to 11 non-self PPIs of the AL and carboxy-terminal RS domain of *hsPrp38* identified in a previous Y2H study (Hegele et al. 2012), we detected 17 additional, nonredundant Y2H interactions using *hsPrp38*^{NTD} and *hsPrp38*^{FL}. Thus, *hsPrp38* might interact with a total of 28 other spliceosomal proteins, representing one of the major PPI hubs of the human spliceosome along with *hshnRNPK* (24 PPIs), *hsRBM4* (24 PPIs), *hsCHERP* (23 PPIs), *hsRBM10* (23 PPIs), and *hsSF3b145* (22 PPIs) (Hegele et al. 2012).

In the majority of known spliceosomal PPIs, the precise interaction interfaces remain unknown. Determining whether the interfaces on a given multi-interaction protein are overlapping or spatially segregated and whether such a protein is capable of binding multiple partners at the same time is important for our understanding of the dynamics of spliceosome assembly. To address this question for *hsPrp38*^{NTD}, we used our structural knowledge and results from our initial Y2H screen to set up alanine surface-scanning two-hybrid arrays, which we evaluated via growth and clustering analyses. This approach allowed us to suggest four binding interfaces for eight of the 12 identified *hsPrp38*^{NTD} interaction partners. Our assignment of interfaces was validated by the observations that (i) a selected interaction (*hsPrp38*^{NTD}-*hsMFAP1*) persisted between the recombinant proteins in SEC, (ii) this interaction was abrogated in an *hsPrp38*^{NTD} variant that also failed to show the original Y2H interaction, and (iii) two proteins with suggested separated interfaces on *Prp38*^{NTD} (*MFAP1* and *Snu23*) can indeed interact with *Prp38*^{NTD} at the same time when using proteins from an organism in which both individual interactions are stable in SEC.

Based on our results, we suggest that *hsPrp38* combines aspects of a date and a party hub. Although several groups of interacting proteins apparently have overlapping binding sites on *hsPrp38* (such as *hsSnu23* and *hsAGGF1*, *hsMFAP1* and *hsSF3b125*, or *hsSnu66*, *hsRBM5*, *hsRBM10*, and *hsSF3b125*), others (such as combinations of individual factors represented in clusters IV–VII) take advantage of different interaction surfaces and may bind concomitantly to *hsPrp38*.

Possible roles of *hsPrp38*^{NTD} in spliceosome assembly

The *hsPrp38*^{NTD} interactors we detected belong to (i) the group of B-specific proteins (*hsMFAP1*, *hsSnu23*, *hsRED*), (ii) proteins of the U4/U6•U5 tri-snRNP (*hsSnu66*, *hsPrp31*), (iii) proteins joining first during the B-to-B^{act} transition (*hsSKIP*, *hsNY-CO-10/hsCwc27*), and (iv) proteins belonging to the groups of hnRNPs (*hsE1B-AP5*), A complex proteins (*hsRBM5*, *hsRBM10*), U2 related proteins (*hsSF3b125*, *hsU2AF35*, *hsCHERP*), or miscellaneous factors associated with the spliceosome (*hsAGGF1*). The first group of interactors (other B-specific proteins) suggests that *hsPrp38*^{NTD} serves to organize at least some of the B-specific proteins as a multimeric complex. This organization could take effect already outside of the spliceosome, allowing the participating proteins to be recruited en bloc. Alternatively, the interactions could emerge only in the context of the spliceosome, with *hsPrp38*^{NTD} serving to spatially arrange its B-specific interactors.

The U4/U6•U5 tri-snRNP also joins the spliceosome during B-complex formation and proteomics analyses showed that its recruitment precedes incorporation of the B-specific proteins (Schneider et al. 2010). Therefore, *hsSnu66* and

hsPrp31 most likely provide additional binding sites for efficient or stable incorporation of *hsPrp38*, and perhaps other B-specific proteins, at the B-complex stage. Notably, because the mapped binding sites for *hsSnu66* (tri-snRNP), *hsMFAP1*, and *hsSnu23* (B-specific) are nonoverlapping on the surface of *hsPrp38*^{NTD}, *hsPrp38* could realize its putative functions as an organizer of a complex of B-specific proteins and as a contact to components of the U4/U6•U5 tri-snRNP at the same time.

We also found Y2H interactions of *hsPrp38*^{NTD} with factors that are not fully present before the B^{act} complex stage (Agafonov et al. 2011)—that is, the Prp19-related protein *hsSKIP* and the peptidyl-prolyl *cis*–*trans* isomerase (PPIase) domain-containing protein *hsNY-Co-10/hsCwc27* (Ulrich and Wahl, 2014). These findings thus suggest that *hsPrp38*, via its NTD, could help to integrate B^{act}-specific proteins into the spliceosome at a transition stage just before *hsPrp38* and other B-specific proteins leave the spliceosome.

Finally, our Y2H interactions also point to potential contacts between *hsPrp38*^{NTD} and factors that join the spliceosome during early stages of assembly (*hsE1B-AP5*, *hsRBM5*, *hsRBM10*, *hsSF3b125*, *hsU2AF35*, *hsCHERP*) (Agafonov et al. 2011). These interactions might help to loosely associate *hsPrp38*, and possibly other B-specific proteins, to early spliceosomal complexes or to recruit these proteins efficiently during B-complex formation. Notably, binding sites of *hsRBM5*, *hsRBM10*, and *hsSF3b125* seem to overlap with the binding site of the tri-snRNP protein *hsSnu66* on *hsPrp38*^{NTD}. Because of the putative, ensuing binding competition, we assume that the early *hsPrp38* partners could initially recruit *hsPrp38* (and perhaps other B-specific proteins) and hand it over to *hsSnu66* after the U4/U6•U5 tri-snRNP has been integrated.

MATERIALS AND METHODS

Plasmids

Open reading frames (ORFs) encoding full-length *hsPrp38*, *hsMFAP1*, and *hsSnu23* were amplified from a human cDNA library and cloned into pETM11 plasmids by EMP cloning (Ulrich et al. 2012). pETM11 plasmids containing synthetic ORFs encoding full-length *C. thermophilum* proteins were obtained from Thermo Fisher Scientific. The pETM11 vector guides production of amino-terminally His₆-tagged, TEV-cleavable fusion proteins. Truncations and point mutations were introduced by inverse PCR as described (Ulrich et al. 2012) or by QuikChange site-directed mutagenesis (Stratagene). All expression constructs were verified by sequencing of the promoter and insert regions.

Protein production and purification

Proteins were produced in *E. coli* Rosetta 2 (DE3) cells either by induction with IPTG (1 mM) in LB medium or by auto-induction in ZY medium (Studier 2005) at 18°C–23°C and for 24–40 h. Selenomethionine-containing *hsPrp38*^{NTD} was produced in M9

medium (2 mM MgSO₄, 0.4% glucose, 0.5 mg riboflavin, 0.5 mg niacinamide, 0.5 mg pyridoxine monohydrate, 0.5 mg thiamine, 50 mg EDTA, 8 mg FeCl₃, 0.5 mg ZnCl₂, 0.1 mg CuCl₂, 0.1 mg CoCl₂, 0.1 mg H₃BO₃, 16 mg MnCl₂, traces of NiSO₄ and molybdcid acid). One liter M9 cultures were grown at 37°C. At OD₆₀₀ 0.5, amino acids (1 g lysine, 1 g threonine, 1 g phenylalanine, 0.5 g leucine, 0.5 g isoleucine, 0.5 g valine, 0.5 g selenomethionine) were added. After an additional 15 min, cultures were shifted to 18°C and induced with 1 mM IPTG. Cells were harvested 48 h post induction.

The following steps were performed at 4°C. Cells were resuspended in solubilization buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 30 mM imidazole, 5 mM β-mercaptoethanol) and disrupted using a Sonopuls Ultrasonic Homogenizer HD 3100 (Bandelin). The soluble fractions were incubated with Ni²⁺-NTA resin (GE Healthcare) for 1 h and washed three times with 10 column volumes of solubilization buffer and then bound protein was eluted with 250 mM imidazole, pH 8.0, 150–500 mM NaCl, and 5 mM β-mercaptoethanol. For removal of the His₆-tag, TEV protease (1:30) was added and samples were dialyzed overnight against 10 mM Tris-HCl, pH 8.0, 150–350 mM NaCl, 30 mM imidazole, and 5 mM β-mercaptoethanol. Instead of TEV protease treatment, selenomethionine-containing *hsPrp38*^{NTD} was digested for 40 h at 4°C by chymotrypsin (1:2000). Cleaved proteins were again passed over Ni²⁺-NTA resin and the flow-through collected. The *hsMFAP1* sample was additionally loaded on a 5 mL HiTrap Q ion-exchange column (GE Healthcare) and eluted via a linear 150–400 mM NaCl gradient. All proteins were concentrated and further purified via Superdex 75 or Superdex 200 size exclusion chromatography (GE Healthcare) in SEC buffer (10 mM Tris-HCl, pH 8.0, 300–350 mM NaCl, 0.1 mM EDTA, and 1 mM DTT). Fractions containing the target proteins were pooled, concentrated, shock-frozen in liquid nitrogen, and stored at –80°C.

Crystallization

All crystals were obtained by sitting drop vapor diffusion with drops containing 1 μL protein and 1 μL reservoir solution at 18°C. Native *hsPrp38*^{NTD} crystals were obtained at a concentration of 12.8 mg/mL with a reservoir solution containing 0.1 M MES, pH 6.5, 15% (v/v) PEG 400 after 1 d. Selenomethionine-containing *hsPrp38*^{NTD} crystals were obtained at 4°C with a protein concentration of 6.8 mg/mL with a reservoir solution containing 0.1 M MES, pH 6.0, 16%–18% (w/v) PEG 4000, 200 mM MgCl₂ after 1 d. *hsPrp38*^{NTD+} crystals were obtained at a protein concentration of 12.0 mg/mL with a reservoir solution containing 0.1 M MES, pH 5.0, 16% PEG 3350, 0.2 M (NH₄)₂SO₄, 5% (v/v) glycerol.

Diffraction data collection and structure determination

For data collection, crystals were flash-cooled in liquid nitrogen after transfer into cryo-protecting solution (crystallization buffer supplemented with 20% (v/v) propylene glycol or 20% (v/v) glycerol). Diffraction data were collected at beamline 14.2 of the BESSY II storage ring (Berlin, Germany) (Mueller et al. 2012) and processed with HKL2000 (Otwinowski and Minor 1997) (*hsPrp38*^{NTD}) or XDS (Kabsch 2010) (selenomethionine *hsPrp38*^{NTD} and *hsPrp38*^{NTD+}). Initial phases for *hsPrp38*^{NTD} were obtained by the SAD strategy

using data collected at the selenium peak wavelength. Five selenium sites were located and used for phasing with the ShelX package (Sheldrick 2008). The experimentally phased map was of high quality and allowed manual building of the structure using COOT (Emsley and Cowtan 2004). The structure of *hsPrp38^{NTD+}* was solved by molecular replacement using MOLREP (Vagin and Teplyakov 2010) from the CCP4 package (Collaborative Computational Project, Number 4 1994) and the structure coordinates of the refined *hsPrp38^{NTD}* model. After placement of the NTD portion, clear negative difference electron density allowed remodeling of the residues 164–179 of *hsPrp38^{NTD+}*. Structural models were completed through alternating rounds of automated refinement using REFMAC5 (Murshudov et al. 2011) or phenix.refine (Afonine et al. 2012) and manual model building using COOT (Emsley and Cowtan 2004). Structure coordinates and diffraction data have been deposited with the Protein Data Bank (<http://www.pdb.org>) under accession codes 4RZ9 (*hsPrp38^{NTD}*) and 4RZA (*hsPrp38^{NTD+}*) and will be released upon publication.

Yeast two-hybrid assays

PPI matrix Y2H screening was performed as described previously (Hegele et al. 2012; Wörseck et al. 2012). Briefly, *PRP38* baits, amino-terminally fused to lexA DNA-binding domains in pBTM116-D9 vectors, were mated in duplicate in 384 array format with prey strains representing approximately 240 spliceosomal proteins by approximately 440 clones (Hegele et al. 2012). Interacting bait-prey pairs were identified by growth on selective agar plates (Leu-Trp-Ura-His). Only bait-prey pairs that showed growth in at least 75% of independent experiments were considered for further evaluation.

Cluster analysis

Interaction mating with variant *hsPrp38^{NTD}* constructs was performed in 96-well MTP format in triplicates. After mating of the bait-prey strains, diploid yeast cultures were first uniformly grown for 2 d on nonselective Leu-Trp agar plates and then transferred to selective Leu-Trp-Ura-His agar plates. Yeast growth after 6 d on selective agar was quantified from grayscale TIFF images by measuring the pixel intensity across the growth area using the AIDA software (raytest). Triplicate values normalized to growth of the corresponding *hsPrp38^{NTD}-hsMFAP1* pair were averaged. Because the D145A/E146A variant of *hsPrp38^{NTD}* showed strongly reduced growth with *hsMFAP1*, normalization was performed for this mutant version using the average size values of unaffected interaction partners. The data matrix of relative yeast growth (rows: *hsPrp38^{NTD}* mutant constructs; columns: interaction partner proteins) was subjected to hierarchical clustering using Euclidean distance.

Analytical gel filtration chromatography

Proteins (50 μ M), alone or as equimolar mixtures, were incubated in SEC buffer for 30 min at 4°C. Fifty microliter samples were analyzed on a Superdex 200 increase 3.2/300 size exclusion column (GE Healthcare) using an ÄKTAmicro system (GE Healthcare) at 4°C. Elution profiles were inspected by SDS-PAGE.

In vitro splicing assays

Pre-mRNA splicing was performed with ³²P-labeled, m7GpppG-capped MINX pre-mRNA in HeLa nuclear extract and analyzed by denaturing PAGE, essentially as described previously (Bessonov et al. 2010), except that the splicing reactions contained 10 nM pre-mRNA. Spliceosomal complexes were analyzed by agarose gel electrophoresis as previously described (Bessonov et al. 2010), and bands were visualized by autoradiography.

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Authors' contributions: All authors designed experiments, analyzed the data, and interpreted the results. T.S., A.K.C.U., L.A., C.L.W., N.B., M.S., and G.W. performed experiments. T.S., A.K.C.U., C.L.W., R.L., U.S., and M.C.W. wrote the manuscript. R.L., U.S., and M.C.W. supervised the project.

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