The pre-mRNA splicing and transcription factor Tat-SF1 is a functional partner of the spliceosome SF3b1 subunit via a U2AF homology motif interface

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© Sarah Loerch 1, Justin R. Leach, Steven W. Horner 2, Debanjana Maji, Jermaine L. Jenkins, Mary J. Pulvino, and Clara L. Kielkopf 3

From the Center for RNA Biology, Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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The transcription elongation and pre-mRNA splicing factor Tat-SF1 associates with the U2 small nuclear ribonucleoprotein (snRNP) of the spliceosome. However, the direct binding partner and underlying interactions mediating the Tat-SF1–U2 snRNP association remain unknown. Here, we identified SF3b1 as a Tat-SF1–interacting subunit of the U2 snRNP. Our 1.1 Å resolution crystal structure revealed that Tat-SF1 contains a U2AF homology motif (UHM) protein–protein interaction module. We demonstrated that Tat-SF1 preferentially and directly binds the SF3b1 subunit compared with other U2AF ligand motif (ULM)-containing splicing factors, and further established that SF3b1 association depends on the integrity of the Tat-SF1 UHM. We next compared the Tat-SF1–binding affinities for each of the five known SF3b1 ULMs and then determined the structures of representative high- and low-affinity SF3b1 ULM complexes with the Tat-SF1 UHM at 1.9 Å and 2.1 Å resolutions, respectively. These structures revealed a canonical UHM–ULM interface, comprising a Tat-SF1 binding pocket for a ULM tryptophan (SF3b1 Trp338) and electrostatic interactions with a basic ULM tail. Importantly, we found that SF3b1 regulates Tat-SF1 levels and that these two factors influence expression of overlapping representative transcripts, consistent with a functional partnership of Tat-SF1 and SF3b1. Altogether, these results define a new molecular interface of the Tat-SF1–U2 snRNP complex for gene regulation.

Tat stimulatory factor 1 (Tat-SF1) 4 was originally identified as a host cofactor that activates Tat-directed HIV-1 transcrip-

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This article contains Table S1 and Fig. S1. The atomic coordinates and structure factors (codes 6N3D, 6N3E, and 6N3F) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 Present address: Janelia Research Campus, Ashburn, VA 20147.
2 Present address: Broad Institute, Cambridge, MA 02142.
3 To whom correspondence should be addressed. Tel.: 585-273-4799; E-mail: clara_kielkopf@urmc.rochester.edu.
4 The abbreviations used are: Tat-SF1, Tat stimulatory factor 1; GST, glutathione S-transferase; ITC, isothermal titration calorimetry; RT-qPCR, quantitative real time reverse transcriptase PCR; snRNP, small nuclear ribonucleoprotein particle; UHM, U2AF homology motif; ULM, U2AF ligand motif; RRM, RNA recognition motif; IP, immunoprecipitation; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.

The pre-mRNA splicing and transcription factor Tat-SF1 is a functional partner of the spliceosome SF3b1 subunit via a U2AF homology motif interface (1, 2). Subsequent studies revealed that Tat-SF1 normally stimulates human transcription elongation (3, 4) as a complex with P-TFb, SPT5, and RAP30 (2, 5–7). The Saccharomyces cerevisiae Tat-SF1 homolog, CUS2, promotes transcription elongation in combination with the RNA unwindases PRP5 and PRP11 (8). Beyond transcription, CUS2 is well-established as an assembly factor for the U2 small nuclear (sn)RNA in the early stages of pre-mRNA splicing (9–13). Like CUS2, human Tat-SF1 influences pre-mRNA splicing (14–18). Reduced Tat-SF1 levels typically promote intron retention rather than exon-skipping or alternative splice sites. Most recently, critical Tat-SF1 functions in pre-mRNA splicing have emerged for embryonic stem cell differentiation (17, 18).

Tat-SF1 co-immunoprecipitates with the U2 small nuclear ribonucleoprotein particle (17–20), which anneals with the pre-mRNA branch point site in the early steps of spliceosome assembly. Yet, the direct interaction partner(s) of Tat-SF1 in the human spliceosome remains unknown. Clues arise from experimental observations coupled with the primary sequences of Tat-SF1 and the U2 snRNP components. Primary sequence analysis suggests that Tat-SF1 contains two modular domains, an RNA recognition motif (RRM) and a U2AF homology motif (UHM) (Fig. 1A). The UHM is a protein-interaction module with specialized features for recognizing short “U2AF ligand motifs” (ULM) of target proteins (21). Among U2 snRNP subunits, SF3b1 singularly comprises ULMs that could mediate Tat-SF1 UHM association; namely, five motifs near the N-terminal region of the SF3b1 protein (Fig. 1, B and C).

Here, we explore the hypothesis that a UHM–ULM interface couples Tat-SF1 to the SF3b1 subunit of the U2 snRNP. We determined a 1.1 Å resolution structure that details the Tat-SF1 UHM-fold. We show that Tat-SF1 directly and specifically recognizes the ULM region of SF3b1, and determine high resolution structures of Tat-SF1 UHM complexes comparing complexes with high- and low-affinity SF3b1 ULMs. The Tat-SF1–SF3b1 ULM interactions are important for association of the proteins in cell extracts. We demonstrate that SF3b1 regulates Tat-SF1 expression levels, and that these splicing factors both regulate representative transcripts. Altogether these results elucidate a functional UHM–ULM-mediated partnership of Tat-SF1 with the SF3b1 subunit of the U2 snRNP spliceosome particle.
UHM-mediated partnership of Tat-SF1 and SF3b1

**Results**

*Tat-SF1 contains a U2AF homology motif*

To confirm that Tat-SF1 contains a UHM and detail its fold, we determined the crystal structure of this region at 1.1 Å resolution (Fig. 2, Table 1). Analysis using the DALI 3D comparison server (22) shows that the structure most closely matches established members of the UHM family, including CAPERα (PDB ID 4OZ1, Z-score 13.3) and U2AF65 (PDB ID 4Z2X, Z-score 13.2). The core βαβαβ-fold also resembles an RRM (e.g. the U1A prototype, PDB ID 3K0J, Z-score 12.8), which shares a common topology and appears to be an ancestor of the UHM. A 310 helix at the N terminus of the Tat-SF1 UHM is stabilized by crystal packing, yet may have relevance in the context of the full-length protein-fold.

The canonical RNA interface of the RRM family involves nucleobase stacking with aromatic residues in two ribonucleoprotein consensus motifs (RNP1 and RNP2) of the β-sheets of the RRM-like fold. In all prior UHM structures, the RNP motifs are degenerate and masked by C-terminal extensions of the core-fold. Accordingly, a C-terminal tail of the Tat-SF1 UHM is stabilized by crystal packing, yet may have relevance in the context of the full-length protein-fold.

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We next tested whether the Tat-SF1 UHM binds a ULM-containing partner (Fig. 3). Most ULM-containing proteins are pre-mRNA splicing factors, including U2AF65 (29), SF1 (30), and SF3b1 (31–33). The inner nuclear protein MAN1 (34) and the poly(Q)-expanded protein ATX1 (35) also contain ULMs. Considering the influence of Tat-SF1 on pre-mRNA splicing (14–18) and its co-immunoprecipitation with the SF3b1-containing U2 snRNP (17–20), we focused on testing Tat-SF1 association with SF3b1 compared with other ULM-containing splicing factors.
Tat-SF1 retained by GST–SF3b1ULM is significant (Fig. 3C). This result demonstrates that Tat-SF1 specifically and directly associates with the ULM region of SF3b1.

Tat-SF1 preferentially binds the fifth SF3b1 ULM

To quantitatively characterize Tat-SF1–SF3b1 interactions, we used isothermal titration calorimetry (ITC) to measure the binding affinities of Tat-SF1 for SF3b1ULM and each of the individual SF3b1 ULMs (Fig. 4, Table 2). Our Tat-SF1 construct for ITC experiments included residues 1–360, which supports snRNP co-immunoprecipitation (19) and avoids precipitation of the UHM fragment during titration. Unlike CAPER/H9251 (23) but similar to U2AF65 (31), the Tat-SF1–SF3b1ULM isotherms show no evidence of cooperative association. We chose boundaries of peptides corresponding to the SF3b1 ULMs to minimize overlap among consecutive motifs (Fig. 1B). Tat-SF1 detectably binds three of the five documented SF3b1 ULMs (ULM1, ULM4, and ULM5). The binding affinity of Tat-SF1 for ULM5 is 20-fold greater than for ULM4 and slightly greater than ULM1 (4-fold). No heats were detected during titrations of Tat-SF1 with ULM2 or ULM3. The apparent binding affinity of Tat-SF1 for the entire SF3b1ULM region agrees with the average values of the highest affinity ULM5 and ULM1 sites (Table 2). We conclude that Tat-SF1 is capable of recognizing three local SF3b1 ULM sites, yet has lower affinity for ULM4 compared with either ULM5 or ULM1.

Structures of Tat-SF1 bound to high and low affinity SF3b1 ULMs

To identify and compare the interactions of the Tat-SF1 UHM with high versus low affinity SF3b1 ULMs, we determined the crystal structures of its complexes with ULM4 and ULM5 at 1.9 Å and 2.1 Å resolutions (Fig. 5, Table 1). Seven residues (residues 291–297) of a 17-residue ULM4 peptide were observed bound to a single Tat-SF1 UHM in the crystallographic asymmetric unit (Fig. 5A). Fewer ULM5 residues are evident (four ordered residues) and one of the two Tat-SF1 UHM copies in the asymmetric unit lacks bound ligand (Fig. 5B). This difference is most likely due to the distinct packing environments of the crystal structures.

The core-fold of the apo-Tat-SF1 UHM is similar to its SF3b1ULM-bound complexes (r.m.s.d. n 0.35–0.45 Å between 86 matching Cα atoms) (Fig. 5C). However, the empty ULM-binding site of the apo-Tat-SF1 structure is filled by a phenylalanine (Phe337) donated by the so-called “RXF” loop of the UHM (21). In the Tat-SF1–ULM complexes, this Phe337 side chain rearranges to enclose the consensus ULM tryptophan.

Table 1

X-ray data collection and refinement statistics

| Tat-SF1 UHM ligand | Apo | ULM4 | ULM5 |
|--------------------|-----|------|------|
| **Data collection** |     |      |      |
| Wavelength (Å)     | 0.83| 1.13 | 0.98 |
| Resolution range (Å) | 30.20–1.13 | 35.05–1.89 | 37.31–2.10 |
| Space group        | P4₁ | P3₁ 21 | P2₁ 21 21 |
| Unit cell lengths (Å) | 30.20, 30.20, 98.74 | 58.37, 58.37, 97.31 | 46.32, 58.71, 125.96 |
| Unit cell angles (°) | 90.90 | 90.90 | 90.90 |
| Total reflections  | 178,395 | 90,661 | 101,323 |
| Unique reflections | 32,586 | 15,748 | 20,337 |
| Multiplicity       | 8.7 (1.9) | 5.7 (1.8) | 5.0 (5.0) |
| Completeness (%)   | 98.8 (99.6) | 99.3 (94.5) | 98.4 (97.0) |
| Mean I/I           | 13.6 (2.9) | 24.3 (2.7) | 10.1 (1.8) |
| Rmerge (%)         | 5.4 (25.2) | 4.4 (45.2) | 8.7 (65.0) |
| R-free (%)         | 3.3 (25.2) | 2.0 (24.3) | 4.3 (36.1) |
| RFO (Å)            | 0.99 (0.84) | 0.99 (0.81) | 0.98 (0.99) |
| **Refinement**     |     |      |      |
| Rwork/Rfree (%)    | 12.7/16.0 | 17.1/19.8 | 17.5/21.9 |
| Tat-SF1 UHM in ASU | 1   | 1    | 2    |
| SF3b1 ULM in ASU  | –   | 1    | 1    |
| No. non-hydrogen atoms |        |      |      |
| Tat-SF1 UHM        | 899 | 799  | 1573 |
| SF3b1 ULM          | –   | 72   | 39   |
| PEG/Glycerol       | 13  | 9    | 13   |
| Ions               | 5   | –    | –    |
| Waters             | 114 | 105  | 151  |
| R.m.s.d. bonds (Å) | 0.007| 0.010| 0.013|
| R.m.s.d. angles (%)| 0.83 | 0.97 | 1.16 |
| Ramachandran (%)   | 98.9/1.1/0 | 100/0/0 | 97.3/2.7/0 |
| Favored/allowed/outlier | 2.76 (93) | 2.93 (99) | 0.94 (100) |
| Clashescore (percentile) | 19.3 | 44.8 | 43.8 |
| (B-factor) (Å²)    | 16.8 | 41.6 | 42.1 |
| SF3b1 ULM          | –   | 69.5 | 89.9 |
| PEG/glycerol       | 45.2 | 61.0 | 80.1 |
| Ions               | 20.7 | –    | –    |
| Water              | 36.4 | 51.3 | 46.5 |
| PDB code           | 6N3D | 6N3E | 6N3F |

Tat-SF1 preferentially binds the fifth SF3b1 ULM

To quantitatively characterize Tat-SF1–SF3b1 interactions, we used isothermal titration calorimetry (ITC) to measure the binding affinities of Tat-SF1 for SF3b1ULM and each of the individual SF3b1 ULMs (Fig. 4, Table 2). Our Tat-SF1 construct for ITC experiments included residues 1–360, which supports snRNP co-immunoprecipitation (19) and avoids precipitation of the UHM fragment during titration. Unlike CAPER/H9251 (23) but similar to U2AF65 (31), the Tat-SF1–SF3b1ULM isotherms show no evidence of cooperative association. We chose boundaries of peptides corresponding to the SF3b1 ULMs to minimize overlap among consecutive motifs (Fig. 1B). Tat-SF1 detectably binds three of the five documented SF3b1 ULMs (ULM1, ULM4, and ULM5). The binding affinity of Tat-SF1 for ULM5 is >20-fold greater than for ULM4 and slightly greater than ULM1 (4-fold). No heats were detected during titrations of Tat-SF1 with ULM2 or ULM3. The apparent binding affinity of Tat-SF1 for the entire SF3b1ULM region agrees with the average values of the highest affinity ULM5 and ULM1 sites (Table 2). We conclude that Tat-SF1 is capable of recognizing three local SF3b1 ULM sites, yet has lower affinity for ULM4 compared with either ULM5 or ULM1.
Integrity of the Tat-SF1 UHM is critical for co-immunoprecipitation with SF3b1

We evaluated the contribution of UHM–ULM interactions to the association of full-length Tat-SF1 and SF3b1 in human cell extracts (Fig. 7). Plasmids expressing Tat-SF1 variants or N-terminally FLAG-tagged SF3b1 were co-transfected into HEK293T cells. The associated protein complexes were immunoprecipitated using Tat-SF1–specific agarose beads. We found that wildtype (WT) Tat-SF1 retained significant levels of FLAG-SF3b1 in lysates from transfected but not mock-transfected cells. This result confirms the existence of a Tat-SF1–SF3b1 complex, in agreement with prior observations in distinct contexts (17–20). We next compared FLAG-SF3b1 co-immunoprecipitants of a Tat-SF1 variant with E286K/F337A mutations, which prevent detectable binding of Tat-SF1 to SF3b1 ULM5 by ITC (Fig. 6D). The mutant Tat-SF1 variant lacked detectable retention of FLAG-SF3b1, which supports the importance of an intact UHM for association of Tat-SF1 with SF3b1 in human cell extracts.

Tat-SF1 expression is sensitive to SF3b1 levels

The association of Tat-SF1 with SF3b1 suggested that its expression level would be mutually regulated to ensure formation of a functional macromolecular complex. To test this hypothesis, we used siRNAs to reduce Tat-SF1 or SF3b1 expression levels in HEK293T cells, and evaluated changes in expression of the other factor by RT-qPCR and immunoblotting (Fig. 8, A–D). Tat-SF1 knockdown had little effect on SF3b1 levels, consistent with prior observations (18). Nevertheless, SF3b1 knockdown significantly reduced Tat-SF1 expression levels. We conclude that whereas SF3b1 serves Tat-SF1-independent functions as a major spliceosome subunit (42), a feedback mechanism modulates Tat-SF1 levels in response to SF3b1.

Tat-SF1 and SF3b1 regulate overlapping gene transcripts

To further explore the functional outcomes of a Tat-SF1 partnership with SF3b1, we evaluated whether SF3b1 regulates expression of Tat-SF1 responsive genes. We focused on three representative transcripts (CCND1, SIN3B, and LMNB1) that
have documented expression changes in response to Tat-SF1 levels (16). As expected, levels of these transcripts decreased following siRNA-mediated reduction of Tat-SF1 (Fig. 8, E–G). Notably, SF3b1 knockdown also decreased the levels of all three Tat-SF1–sensitive transcripts (by 3–5-fold). These effects are likely to be direct, because SF3b1 expression is only subtly affected by Tat-SF1 levels (Fig. 8, B and C) (18). Despite numerous studies (e.g. Refs. 14, 16, and 18), to our knowledge neither we nor others have succeeded in rescuing gene expression by reintroducing Tat-SF1, which suggests that Tat-SF1 activities are delicately balanced by localization and/or post-translational modifications. To reinforce the functional convergence of Tat-SF1 and SF3b1, we compared an additional transcript (DUSP11) that is known to be sensitive to an SF3b1-targeted small molecule, spliceostatin-A (43). Both SF3b1 and Tat-SF1 knockdown decreased levels of the DUSP11 transcript (Fig. 8H).

As a preliminary check for alternative splicing, we compared the sensitivity of a distinct splice site from LMNB1 to Tat-SF1 and SF3b1 knockdown (Fig. S1). We observed similar changes in transcript levels when probing either LMNB1 splice site. Consistently, Tat-SF1 reduction typically leads to intron retention rather than alternatively splicing (17, 18), and most intron-retained transcripts would be removed by quality control pathways (44). As such, our experiments lack discrimination between altered transcription and splicing. Regardless, the significant impact of SF3b1 knockdown for all Tat-SF1–sensitive transcripts supports altered pre-mRNA splicing, considering the well-established roles of SF3b1 in the spliceosome (42).

| Interaction                        | K_D  | ΔH   | ΔS   |
|------------------------------------|------|------|------|
| Wildtype Tat-SF1 titrated with ULM regions |      |      |      |
| SF3b1ULM                           | 1.8 ± 0.9 | -66.1 ± 3.0 | 58.0 ± 3.0 |
| ULM1                               | 3.0 ± 0.5 | -11.8 ± 0.3 | 4.1 ± 0.3  |
| ULM2 b                             | No detectable binding | | |
| ULM3 b                             | No detectable binding | | |
| ULM4 b                             | 18.9 ± 4.7 | -34.1 ± 25.3 | 27.5 ± 25.5 |
| ULM5                               | 0.8 ± 0.1 | -18.7 ± 1.0 | 10.2 ± 1.2  |
| E286K/D290K mutant Tat-SF1 titrated with ULM5 |      |      |      |
| No detectable binding            |      |      |      |
| E286K/F337A mutant Tat-SF1 titrated with ULM5 |      |      |      |
| No detectable binding            |      |      |      |

* Calculated using ΔG = ΔH-ΔS, with ΔG = -RT ln (K_D) and T = 303 K.
* No heats detected during titration of 200 μM ULM into 20 μM Tat-SF1 protein.
* Values are estimates due to low binding affinity.
And 46–48

SF3b1 association further depends on electrostatic interactions SF3b1 ULM between two motifs. The Tat-SF1 UHM encloses a central tryptophan of the extended C terminus that masks degenerate ribonucleoprotein proteins. Our high resolution structures reveal that the Tat-SF1 face for Tat-SF1–SF3b1 association in cell extracts or purified structural homology, we pinpoint a critical UHM–ULM inter-

Tat-SF1 to stimulate transcription (Fig. 5, A and D). Following clues from structural homology, we pinpoint a critical UHM–ULM inter-

action affinities of the Tat-SF1–SF3b1 protein domains are micro-
molar (Table 2), consistent with other SF3b1 ULM–UHM pro-

tein complexes (21). Such relatively weak interactions could be important for dynamic associations (45), yet do not rule out enhancement by transient interfaces during spliceosome assembly. For example, the UHM-mediated association of Tat-SF1 with the SF3b1 ULMs may synergize with the U2 snRNA, which Tat-SF1 and SF3b1 bind in the context of the intact U2 snRNP. Indeed, an RRM mutation of the yeast homologue Cus2 prevents its U2 snRNA association and rescue of U2 snRNA misfolding (9, 19).

Macromolecular complexes such as spliceosome particles often employ feedback mechanisms to coordinate subunit expression (e.g. Refs. 46–48). Our finding that Tat-SF1 expression decreases following SF3b1 depletion (Fig. 8, A and D) supports functional coordination of Tat-SF1 and SF3b1. Conversely, SF3b1 levels are relatively insensitive to reduced Tat-SF1, which agrees with SF3b1 ULM associations with a variety of alternative splicing factors (23, 26, 27, 31). The SF3b1-dependent Tat-SF1 expression is analogous to a previously characterized precedent, the U2AF heterodimer (46). U2AF65 knock-down reduces U2AF35 protein levels, whereas reduced U2AF35 levels are insensitive to U2AF65. Functionally, in vitro splicing can be reconstituted by the U2AF65 subunit alone (49–51), and moreover, Schizosaccharomyces pombe introns that are affected by U2AF65 depletion are affected by U2AF35 depletion (52). Accordingly, we here find that the levels of representative downstream transcripts respond to both Tat-SF1 and SF3b1 reductions. These effects are likely to be specific, because SF3b1 (43, 53, 54) and Tat-SF1 (16, 17, 55) knockdowns alter expression and splicing of only a subset of genes. We suggest that a feedback mechanism promotes the formation of Tat-SF1 complexes with SF3b1.

Altogether, we have identified SF3b1 as a directly interacting partner of Tat-SF1, and detailed a requisite ULM–UHM interface of this functional complex at atomic resolution. Beyond our study of Tat-SF1, the SF3b1 ULM-binding preferences have been comprehensively determined for one other UHM-containing protein, U2AF65 (31). Both Tat-SF1 and U2AF65 bind the SF3b1 ULM region with similar apparent affinities (approximate $K_d$ values 2 μM/3 μM for Tat-SF1/U2AF65 binding to SF3b1ULM), and both show a subtle binding preference for ULM5 among the SF3b1 ULMs. Immunoaffinity purification followed by MS has shown that Tat-SF1 associates with U2 snRNP particles, but is absent from assembled, “A”-stage spliceosomes (20). In contrast, U2AF65 is an essential splicing factor that stabilizes U2 snRNP association with pre-mRNA splice sites (56, 57), and is detected only in the A- and later “B”-stages of spliceosome assembly (20). The similar SF3b1 binding preferences of Tat-SF1 and U2AF65, coupled with sequential Tat-SF1 and U2AF65 functions during transcription and spliceosome assembly, call for future studies to explore whether Tat-SF1 serves as a checkpoint factor for handoff of functional U2 snRNP to U2AF65 during spliceosome assembly.

**Experimental procedures**

**Recombinant protein expression and purification**

Human Tat-SF1 (residues 1–360 of NCBI Refseq NP_055315), Tat-SF1 UHM (residues 260–333), SF3b1ULM (residues 190–
344 of NCBI RefSeq NP_036565), U2AF65 (residues 85–475 of NCBI RefSeq NP_009210), or SF1 (residues 1–255 of NCBI RefSeq NP_004621) were expressed in *Escherichia coli* as GST fusions using either pGEX-6p or an in-house pGEX-6p variant encoding a tobacco etch virus protease site. The splicing factor proteins were purified by GST affinity followed by anion exchange or heparin affinity chromatography. For crystallization experiments, the Tat-SF1 UHM was separated from aggregates by size exclusion chromatography in 50 mM NaCl, 15 mM HEPES, pH 7.4, 0.2 mM tris(2-carboxyethyl)phosphine. Purified SF1 proteins were phosphorylated by UHMK1 as described (25) and then separated from UHMK1 by size exclusion chromatography. Synthetic peptides corresponding to SF3b1 ULMs (with residue ranges shown in Fig. 1) were purchased at 98% purity (Biomatik or Bio-Synthesis, Inc.).

**GST pulldown assays**

The GST fusion “bait” protein (100 µg) was incubated for 1 h on pre-packaged GSH-agarose spin columns with 0.2-ml bed volume (Thermo Scientific Pierce™) that were equilibrated three times with binding buffer (100 mM NaCl, 25 mM HEPES, 0.2 mM tris(2-carboxyethyl)phosphine. The GST fusion proteins were incubated with overnight incubation and washed to remove unbound material. The GST fusion proteins were eluted with 200 µl of 150 mM glutathione in binding buffer. The eluted GST fusion proteins were separated on a 10% SDS-PAGE gel and visualized by Coomassie blue staining.
pH 7.4, 3% glycerol, 1 mM EDTA, 10 mM DTT), and supplemented with protease inhibitor. The immobilized GST fusions were incubated with purified protein (500 μg, 1 mg/ml) in binding buffer for 45 min at 4 °C. The resin was then washed five times with binding buffer. Retained proteins were eluted by heating in Laemmli loading buffer supplemented with 10 mM GSH, and analyzed by SDS-PAGE with Coomassie Blue staining.

Isothermal titration calorimetry

Protein samples were dialyzed into 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.2 mM tris(2-carboxyethyl)phosphine, and peptides were diluted into >200-fold greater volume of dialysis buffer, prior to ITC. The thermodynamics of Tat-SF1 titrated with SF3b1 ULm or ULM peptides were measured using a VP-ITC and fit using Origin 7.0 (MicroCal, LLC). Control titrations of ULM peptides into buffer were linear and showed negligible heats of dilution, and were subtracted to correct the relatively low affinity isotherms of ULM1 and ULM4. Other isotherms were corrected for the heats of dilution using an average of three data points at the saturated plateau of the titration. The apparent stoichiometries are consistent with 1:1 binding except SF3b1 ULm, which is underdetermined and appears to bind between two and five Tat-SF1 molecules considering χ² values of different fits.

Crystallization and structure determination

For crystallization of the Tat-SF1 UHM–SF3b155 ULM4 and ULM5 complexes, 0.1 mM Tat-SF1 UHM was mixed with a 1.2-fold molar excess of SF3b155 ULM and concentrated in a 2-kDa MWCO Vivaspin Hydrosart (Sartorius) concentrator. The Tat-SF1 UHM and its ULM complexes were crystallized by vapor diffusion using the JCSG TM screen (Molecular Dimensions) at 277 K. The apo-Tat-SF1 UHM crystallization condi-

Figure 8. Tat-SF1 expression levels decline following SF3b1 depletion and in turn decrease expression of similar representative transcripts as SF3b1. A and B, immunoblots demonstrating Tat-SF1 or SF3b1 knockdown following transfection of HEK293T cells with the indicated StealthTM siRNAs or a low G/C control siRNA (“low GC”). Whole cell lysates were analyzed by SDS-PAGE and immunoblotted for either: A, Tat-SF1 or B, SF3b1. H2B is encoded by an intronless gene and hence an appropriate loading control. C–H, relative levels of Tat-SF1, SF3b1, or the indicated transcripts were determined by RT-qPCR of total RNA isolated from HEK 293T cells following transfection with the indicated siRNAs. Amplification of the LMNB1 exon 5/6 junction is shown. The LMNB1 exon 7/8 junction gave similar results as shown in Fig. S1. Primer locations and sequences are given in Table S1. Unpaired, two-tailed t tests with Welch’s correction: *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.0001.
tions were optimized in hanging drop format to 0.4 mM MgCl₂, 0.1 mM Tris, pH 7.5, 15% PEG 8000. The respective crystallization solutions of ULM4 and ULM5 complexes contained 0.2 mM magnesium formate, 20% PEG 3350, and 0.1 mM Tris, pH 8.5, 15% PEG 8000. The crystals were sequentially transferred to 20% glycerol in synthetic mother liquor then flash-cooled in liquid nitrogen. Crystallographic data sets were collected remotely at Stanford Synchrotron Radiation Lightsource beamlines 12-2, 7-1, and 9-2 (58). Data were processed using the SSRL AUTOXDS script (A. Gonzalez and Y. Tsai) implementation of XDS (59) and CCP4 packages (60).

The apo-Tat-SF1 UHM structure was determined with an ensemble search model comprising the U2AF₆₅, SF45, and CPERe UHM structures generated using Ensembler (61). A molecular replacement solution using Phaser (61) via the Phenix interface (62) (TF Z-score 5.8, LLG 304) was rebuilt in Coot (63), and refined using Phenix (62). Alternative strategies to determine the apo-Tat-SF1 UHM structure were unsuccessful, including direct methods at 1.13 Å resolution and molecular replacement searches with either individual UHM crystal structures stripped of ligand coordinates (U2AF₆₅, PDB ID 4FXW; SF45, PDB ID 2PEH; CPERe, PDB ID 4OZ0) or an unpublished apo-NMR structure (PDB ID 2DIT). The ULM-bound structures were determined by molecular replacement starting from the apo-Tat-SF1 UHM structure. Crystallographic data and refinement statistics are given in Table 1.

Cell culture and transfections

HEK 293T cells (ATCC® CRL-3216™) were cultured in Gibco™ minimum essential media supplemented with 10% fetal calf serum (Atlanta Biologicals) and 2 mM L-glutamine and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were transfected using JetPrime® (Polyplus-transfection®) as instructed by the manufacturer.

Co-immunoprecipitation

HEK 293T cells were transfected with plasmids encoding human Tat-SF1 (modified from Origene #SC114990), N-terminally FLAG-tagged SF3b1 (modified from a gift of Benjamin Ebert (64)), or empty control vector (pCMV). After 24 h, cells were harvested and lysed in a IP buffer (50 mM Tris, pH 80.0, 75 mM NaCl, 5% glycerol, 10 mM CaCl₂, cOmplete™ EDTA-free protease inhibitor (Sigma), 0.5 mM DTT) + 0.5% Triton X-100. Lysates were clarified by centrifugation and a fraction of each sample was set aside as input controls. The remaining lysates were diluted 1:5 with IP buffer + 0.1% Triton X-100, then incubated for 1 h on a rocking platform at 4 °C protein G-Sepharose (GE Healthcare number 17061801) pre-bound to Tat-SF1-specific antibody (Santa Cruz Biotechnology number sc-514351). Beads were collected by centrifugation and washed extensively with IP buffer. Final washes included RNase A (Sigma).

Immunoblotting

The immunoprecipitated complexes on beads were resuspended in SDS-PAGE loading buffer. For total protein analysis, cells were lysed in a buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM DTT, and protease inhibitors. Proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with antibodies specific for Tat-SF1 (Santa Cruz Biotechnology number sc-514351), FLAG (Cell Signaling Technology number 2368), SF3b1 (MBL number D221-3), or Histone H2B (Cell Signaling Technology number 12354). Secondary antibodies included anti-mouse IgG horseradish peroxidase or anti-rabbit IgG horseradish peroxidase (GE Healthcare numbers NA931 or NA934). Chemiluminescent signal from SuperSignal WestPico chemiluminescent substrate (ThermoFisher Scientific) was detected on a Chemidoc™ Touch Imaging System (Bio-Rad).

Quantitative-reverse transcriptase real time PCR

Cells were harvested 3 days following transfection with Stealth™ siRNA duplexes (ThermoFisher Scientific) specific for HTATSF1 (assay IDs: HSS120650, HSS120651), SF3B1 (assay IDs: HSS146413, HSS146415), or a negative control (12935200). Total RNA was isolated and DNase I-treated using the RNeasy Kit (Qiagen). The cDNAs were synthesized by Moloney murine leukemia virus RT with random primers (Invitrogen), and analyzed by quantitative PCR with SYBR™ Green in triplicate on a Bio-Rad CFX thermal cycler. The RT-qPCR products were quantified by the relative standard curve method and normalized to the level of 18S rRNA. Results in Figs. 7 and 8 represent at least three biological replicates. Primers are listed in Table S1.

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