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Supplemental Information

Component of splicing factor SF3b plays a key role in translational control of polyribosomes on the endoplasmic reticulum

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials and Methods

Plasmids and cDNA amplification

Using total mRNAs from HEL cells as templates, cDNAs were obtained by PCR amplification of two human genes, SF3b4 (GenBank No. NP_005841) and COL1A1 (GenBank No. NM_000088) and subcloned to generate mammalian expression plasmids. The SF3b4 gene was subcloned into pEF-MycHis (Invitrogen) to generate the expression plasmid pEF-SF3b4.The obtained plasmids were verified by DNA sequencing. Expression plasmids for wild-type and mutant forms of human p180 were prepared as described previously (1). The cDNA of secreted AP (GenBank No. NP_001623.3) was PCR-amplified using AP-tag4 (Invitrogen) as a template and subcloned into the Nhe I-Xho I site of a mammalian expression plasmid (pEGFP-C3; Clontech). The obtained plasmid, prCMV-SEAP, contained an expression unit for the secreted form of AP driven by the CMV promoter with a polyA signal, but no EGFP-coding region. Restriction enzyme sites for Bgl II and Hind III were introduced between the promoter and the initiation codon to evaluate test sequences. The full-length cDNA of human *COL1A1* (5297 bp) was subcloned into prCMV-SEAP to generate prCMV-fullCOL1A1. prCMV-COL1A1ORF (127–4251) and prCMV-COL1A1 ORF-3′UTR (127–5297) were constructed in a similar manner. The cDNAs encoding the 5′ UTR sequences for human *FN1* (266 bp) (GenBank No. NM_212482.1) and human *CANX* (177 bp) (GenBank No. NM_001746.30) were amplified by RT-PCR and subcloned into prCMV-SEAP. The sequences in all plasmids were verified by DNA sequencing.

Antibodies

The following antibodies were used: rabbit antibodies against GFP (Clontech), calnexin (Stressgen), human p180 (N1), $(His)_5$ tag (Qiagen), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling), Sec61 beta (Upstate), Sec61alpha and TRAP alpha (generous gifts from C.V. Nicchitta, Duke University Medical Center); mouse mAbs against human type I procollagen (SP1.D8; Developmental Studies Hybridoma Bank, University of Iowa), and goat antibodies against SF3b4 (Santa Cruz Biotechnology), eukaryotic elongation factor 1a (eEF1a; Santa Cruz Biotechnology), splicing factor 1 (SF1; Abgent), coiled-coil domain-containing protein 50 (CCDC50; Abgent), and lamin A/C (LMNA; GeneTex).

Cell culture

Culture of HEL fibroblasts was carried out as previously described (1). Ascorbic acid phosphate magnesium salt *n*-hydrate (0.2 mM, Wako) was added to the cells to promote procollagen folding and secretion. A siRNA for human SF3b4 (HSS115684; Life Technologies) and a control siRNA (Stealth™ RNAi Negative Control Duplex; Invitrogen) were used.

Affinity column chromatography of histidine-tagged Ct

Hexahistidine-tagged Ct protein (His-Ct) was overexpressed in HEL cells. Cytosolic fractions prepared after *in situ* permeabilization were subjected to affinity chromatography using TALON (Clontech). The buffers for the equilibration, washing, and elution steps contained 25 mM Hepes-KOH (pH 7.5), 110 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM EGTA, 0.015% digitonin, and protease inhibitor mixture (EDTA-free Complete Inhibitor Mixture; Roche Applied Science). After extensive washing in 5 mM imidazole-containing buffer, captured His-Ct protein was eluted using 50 mM imidazole-containing buffer and fractionated into four fractions (E1–E4). Western blotting analysis showed that most of the His-Ct was eluted in fractions E1, E2 and E3 (see fig. S2*A*), whereas no signal was detected in mock-transfected cells. The proteins of E1, E2 and E3 fractions were identified by LC-MS/MS after trypsin digestion as described previously (2).

Tandem mass spectrometry (MS/MS) analyses for identifying proteins in the purified Ct fractions

The purified fractions were dissolved in 100 mM ammonium bicarbonate and reduced with 10 mM dithiothreitol at 56°C for 30 min, followed by alkylation of the cysteine residues using 25 mM iodoacetamide at room temperature for 30 min in the dark. The samples were digested with sequencing grade modified trypsin (Promega) at 37°C for 16 h. The sample solutions were then acidified with formic acid and the proteins were identified using a mass spectrometer (3200 QTRAP; AB Sciex) coupled to an HPLC system (Agilent 1200 Series; Agilent Technologies). The peptide mixtures were separated on an Ascentis Express C18 column (2.7-μm particle size; L × I.D. 150 mm × 2.1 mm; Supelco). The eluted peptides were analyzed using an information-dependent acquisition method operated by selecting the two most intense precursor ions in a prior survey MS scan and then subjecting the precursor ions to MS/MS fragmentation. The collision energy was automatically determined based on the mass and charge state of the precursor ions using rolling collision energy. The MS and MS/MS scans were obtained over the *m/z* ranges of 400–1300 and 100–1700, respectively. The acquired MS/MS spectra were searched against the UniProtKB/Swiss-Prot database (release 2011_08, July 2011) for *Homo sapiens* (20244 protein entries)

using ProteinPilot software 4.0 (AB Sciex) with the Paragon™ algorithm. Search parameters included digestion by trypsin, cysteine alkylation by iodoacetamide, biological modifications ID focus, and 66% protein confidence threshold. Default parameters, including the number of missed cleavages permitted and the mass tolerance for precursor ions and fragment ions, were adopted by the software.

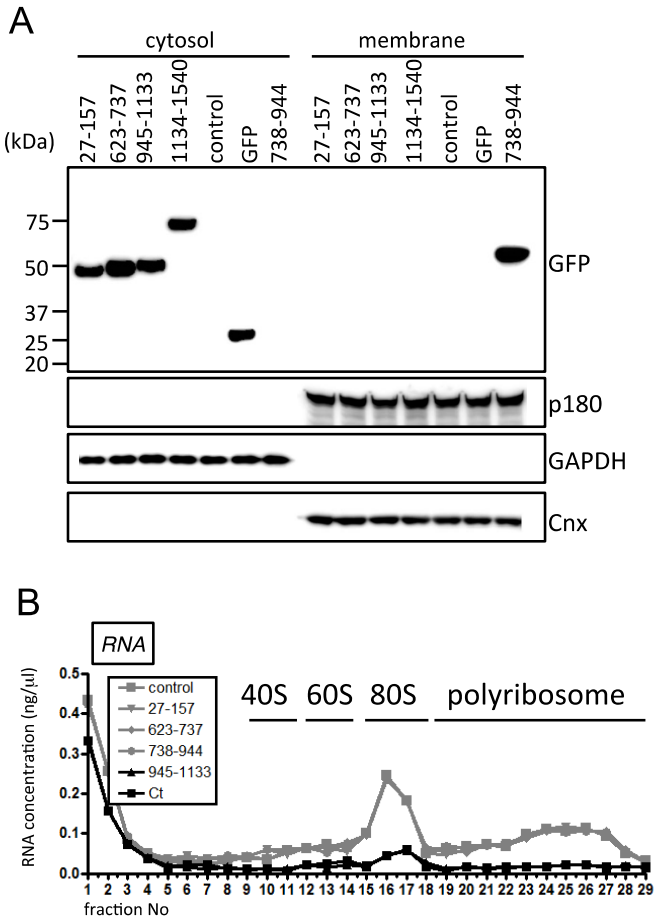


fig. S1. Related to Figure 1.
(*A*) A series of GFP-tagged polypeptides containing different regions of human p180 were expressed in HEL cells and their cytosolic and membrane fractions were prepared by sequential detergent extraction. Each preparation was verified by marker protein detection using western blotting analyses, i.e., GAPDH for cytosolic fractions and calnexin for membrane fractions. The examined proteins were expressed with the correct sizes in the cytosol. (*B*) Total RNA profiles of the fractionated samples depicted in Fig. 1*D* are shown.

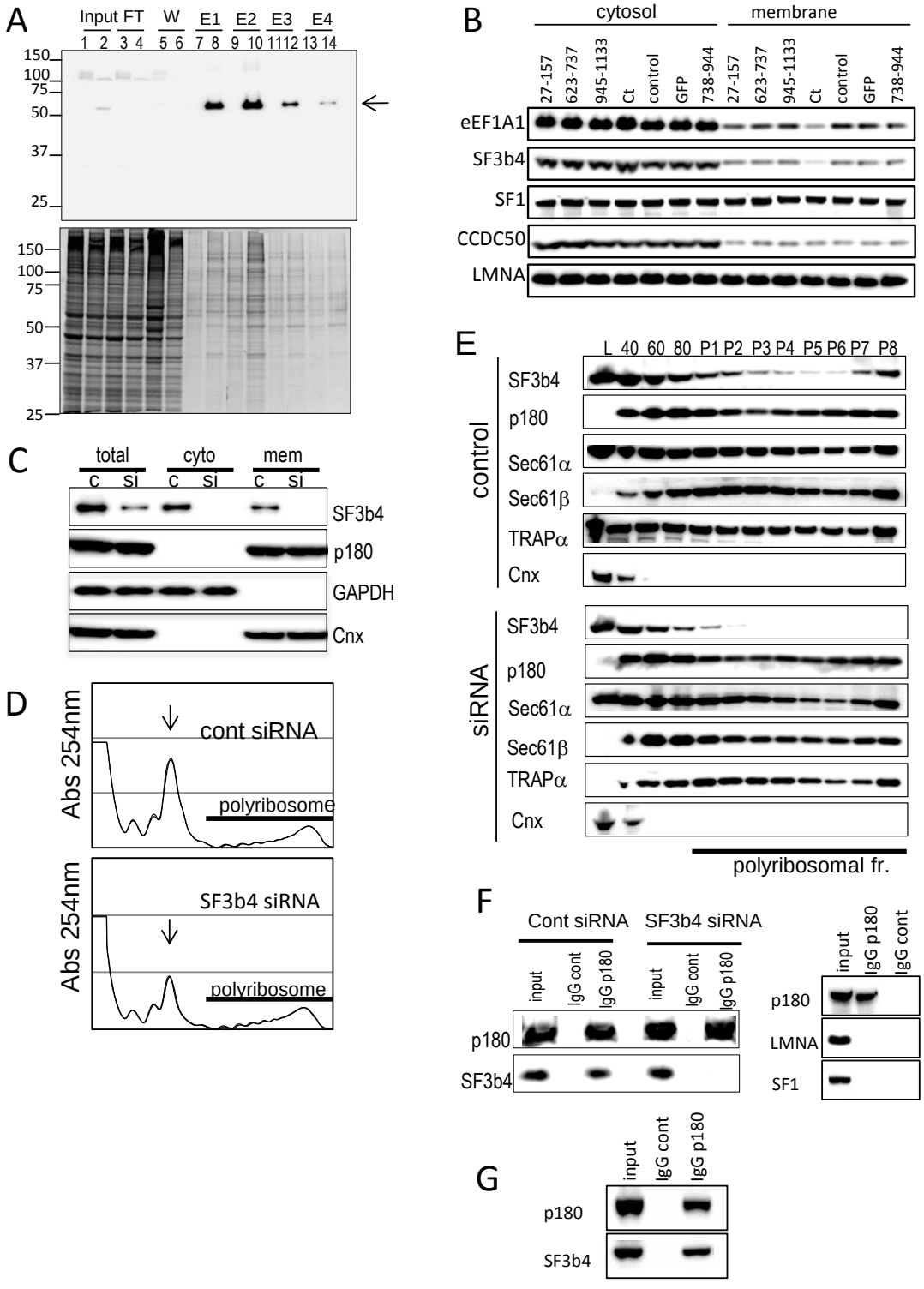


fig. S2. Related to Figure 2**.**

(*A*) Ascorbate-treated HEL cells were transfected with hexahistidine-tagged Ct (even number lanes) or mock-transfected (odd number lanes) and the cytosolic fractions were subjected to Talon affinity column chromatography (FT: flow-through fraction; W: wash fraction: E1–E4:

elution fractions with 50 mM imidazole-containing buffer). The eluted Ct detected using an anti-$(His)_5$ tag antibody by western blotting is shown with an arrow (upper panel). Protein staining of the gel with Oriole (QIAGEN) is shown in the lower panel. (*B*) The cytosolic and membrane fractions of GFP-tagged polypeptides expressed in HEL cells were analyzed by western blotting. (*C*) Total cell lysates, cytosolic fractions, and membrane fractions from HEL cells treated with control siRNA or siRNA specific for human SF3b4 were analyzed by western blotting to monitor cytoplasmic (GAPDH) and membrane (calnexin (Cnx), p180) marker proteins. (*D*) Membrane fractions of HEL cells treated with control siRNA or SF3b4-specific siRNA were subjected to sucrose density gradient centrifugation and their RNA profiles were monitored at 254 nm. Arrows indicate peaks corresponding to the 80S ribosome. (*E*) Western blot analysis of translocon components in the membrane fractions after sucrose density gradient centrifugation, as used in (*D*). (*F*) The membrane fractions of HEL cells treated with control siRNA or siRNA against SF3b4 were immunoprecipitated with an anti-p180 antibody or control IgG. Specific immunoprecipitation with the anti-p180 antibody was verified by western blotting (left). Specificity of the immunoprecipitation was confirmed by using antibodies against the negative control proteins, LMNA and SF1. These proteins were not immunoprecipitated when subjected to the same protocol (right). (*G*) The membrane fractions were treated with S1 nuclease (New England Biolabs) at 37°C for 15 min. After adding EGTA (final conc. 5 mM), immunoprecipitation with the anti-p180 antibody was performed. Ribonuclease treatment failed to disturb SF3b4-p180 interaction, indicating that this interaction is not dependent on RNA.

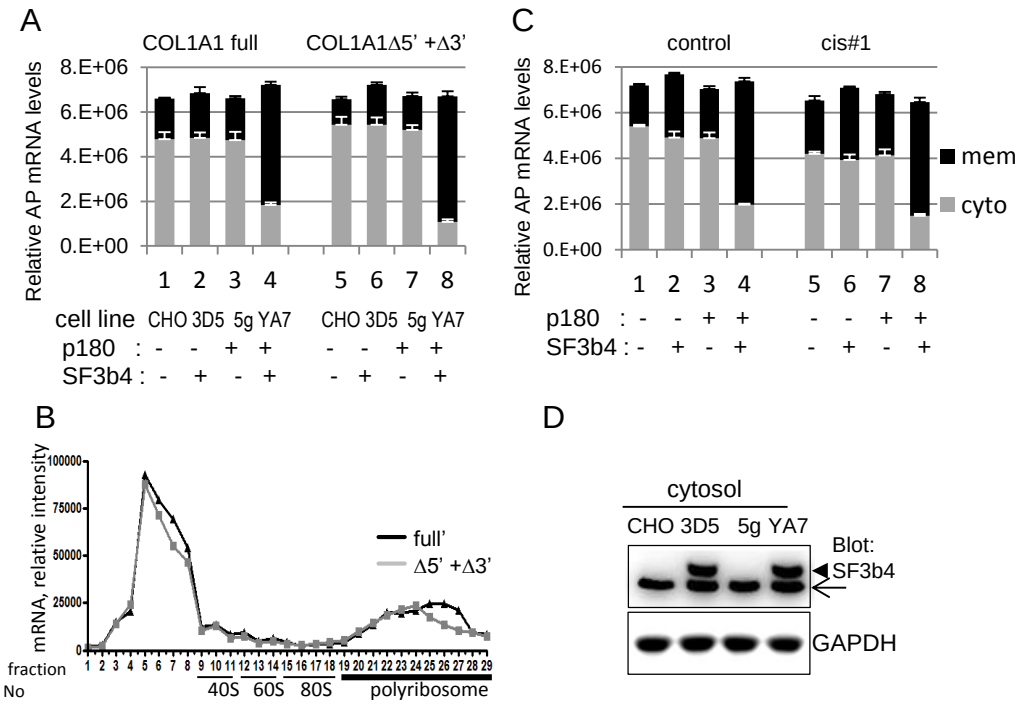


fig. S3. Related to Figure 3 and Figure 4.

(*A*) The four indicated cell lines were transfected with full-length (left) or a mutant Δ5 + Δ3′ *COL1A1* cDNA as in Fig. 3*B*, and cytosolic and membrane fractions were prepared. Relative *COL1A1* mRNA expression levels were estimated by qPCR. Data represent means ± SD (n = 3 or 4). (*B*) Polyribosomal profiles of *COL1A1* cDNA of the membrane fractions of YA7 cells after sucrose density gradient centrifugation are shown. The cells had been transfected with expression plasmids encoding full-length (black) or mutant Δ5+ Δ3′ (grey) *COL1A1* cDNA. A clear peak shift was observed for full-length *COL1A1* cDNA. (*C*) The four cell lines were transfected with reporter plasmids and their relative amounts of cDNA encoding secreted *AP* mRNA are shown as in (*A*). Columns 1–4: control reporter plasmid without the *cis*-element; columns 5–8: reporter plasmid containing the cis#1 element upstream of the initiation codon. Data represent means ± SD (n = 3). (*D*) Western blotting analyses of cytosolic fractions of the indicated cell lines are shown. YA7 and 3D5 cells showed similar levels of cytosolic SF3b4. An arrow represents positions for endogenous SF3b4, while an arrowhead is that of recombinant (myc-tagged) SF3b4.

A

cis#1-sub(T):
TCGTCG**TTTTTTTTT**GGAGTTTCTCCTCGGGGTCGGAGCAGGAGGCACGCGGAGTGT**TTTTTTTTT**CAT**T**
TTTTTTTTCTAACCCCCTCCCCAGCCACAAA**TTTTTTTTT**TGTCTAGGGTCTAGAC

cis#2-sub(T):
GCCCGCGCCGGCTGTGCTGCACAGGGGGAGGA**TTTTTTTTT**CCAGGCGCGAGCGGGAA**TTTTTTTTT**TGC
AGCCACAACTTCTCTGGTCCTCTGCATCCCTTCTGTCCCTCCACCCGTCCCCTTCCCCACCCTCTGGCCC
CCACCTTCTTG**TTTTTTTTT**ACCCCCGGGAGGCATTAGAAGGGATTTTTCCCGCAGGTTGCGAAGGGAAG
CAAACTTGGTGGCAACTTGCCTCCCGGTGCGGGCGTCTCTCCCCCACCGTCTCAAC

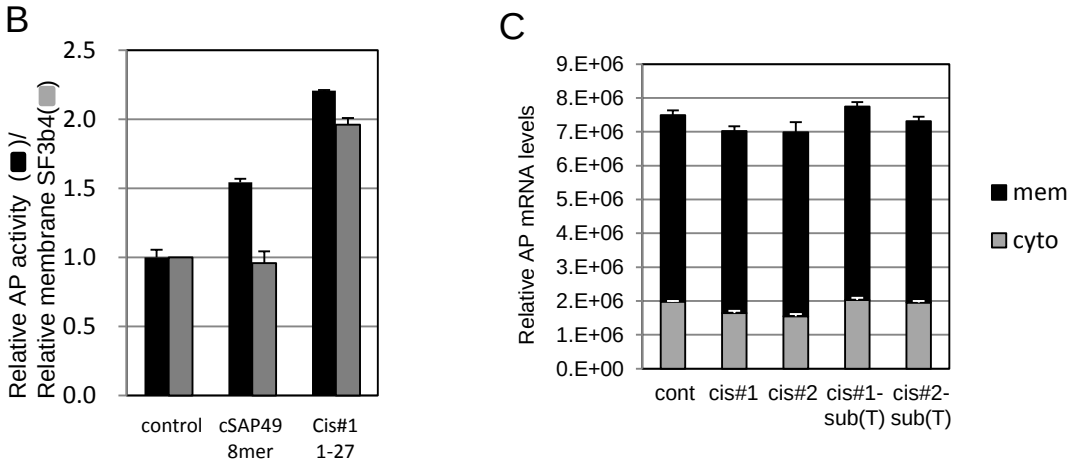
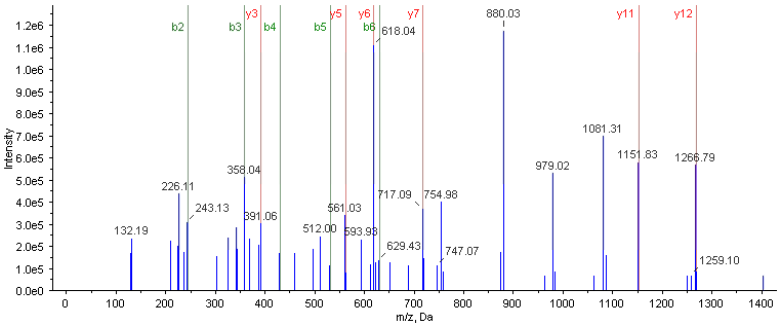


fig. S4. Related to Figure 5.
(*A*) Sequence of the mutated cis#1 and cis#2 elements lacking the motifs (used in Fig. 5*C* and *D*). (*B*) YA7 cells were transfected with reporter plasmids containing nt 1–27 of cis#1 or the octamer CGUGUGAG. Relative AP activities in samples of medium (black bars) and the relative amount of membrane SF3b4 (gray bars) estimated by densitometric scanning of western blots at 18 h post-transfection are shown. The values in control experiments using empty reporter plasmid were set as 1. Data of relative AP activity represent means ± SD (n = 3). (*C*) Relative *AP* mRNA levels of the membrane and cytosol fractions of cells in Fig. 5*D* are shown. Data represent means ± SD (n = 3 or 4).

A

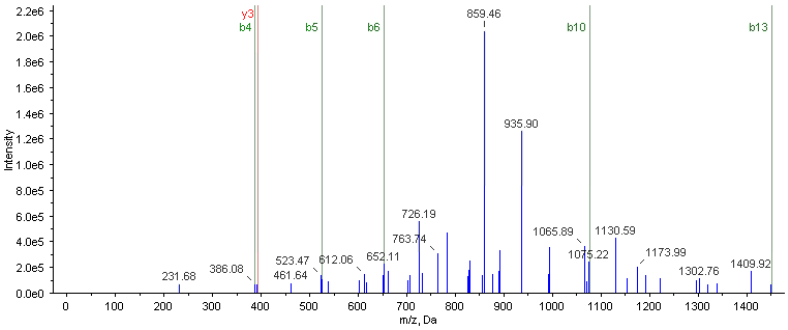
NQDATVYVGGLDEK



	b	y
N	115.05	1508.72
Q	**243.11**	1394.68
D	**358.14**	**1266.62**
A	**429.17**	**1151.59**
T	**530.22**	1080.56
V	**629.29**	979.51
Y	792.35	880.44
V	891.42	**717.38**
G	948.44	**618.31**
G	1005.46	**561.29**
L	1118.55	504.27
D	1233.57	**391.18**
E	1362.62	276.16
K	1490.71	147.11

B

VTGQHQGYGFVEFLSEEDADYAIKIMNMIK



	b	y
V	100.08	3433.64
T	201.12	3334.58
G	258.14	3233.53
Q	**386.20**	3176.51
H	**523.26**	3048.45
Q	**651.32**	2911.39
G	708.34	2783.33
Y	871.41	2726.31
G	928.43	2563.25
F	**1075.50**	2506.22
V	1174.56	2359.16
E	1303.61	2260.09
F	**1450.68**	2131.05
L	1563.76	1983.98
S	1650.79	1870.89
E	1779.83	1783.86
E	1908.88	1654.82
D	2023.90	1525.78
A	2094.94	1410.75
D	2209.97	1339.71
Y	2373.03	1224.68
A	2444.07	1061.62
I	2557.15	990.58
K	2685.25	877.50
I	2798.33	749.40
M	2929.37	636.32
N	3043.41	505.28
M	3174.45	**391.24**
I	3287.54	260.20
K	3415.63	147.11

fig. S5. MS/MS spectra of tryptic peptides. Related to Figure 2 and Table S1.

(*A*) NQDATVYVGGLDEK (*m/z* 754.86, *z* = 2) and (*B*) VTGQHQGYGFVEFLSEEDADYAIKIMNMIK (*m/z* 859.16, *z* = 4) of SF3b4 are shown.

Table S1. Summary of identified proteins that were specifically co-purified with the Ct protein

Related to Figure 2 and figure S2.

Fraction	N	Score	Coverage (%)	Accession number	Protein	Membrane localization*
Ct_E1	1	6.2	9.7	sp|Q9P2E9|RRBP1_HUMAN	Ribosome-binding protein 1	Recombinant Ct derived
	2	4.64	8.3	sp|Q68EM7|RHG17_HUMAN	Rho GTPase-activating protein 17	−
	3	4	6.4	sp|P08670|VIME_HUMAN	Vimentin	−
	4	0.52	5.3	sp|O75083|WDR1_HUMAN	WD repeat-containing protein 1	−
Ct_E2	1	8.03	42.4	sp|Q9P2E9|RRBP1_HUMAN	Ribosome-binding protein 1	Recombinant Ct derived
	2	4.08	24.2	sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	+
	3	**3.53**	**28.8**	**sp|Q15427|SF3B4_HUMAN**	**Splicing factor 3B subunit 4**	**+**
	4	2.86	14.5	sp|P02545|LMNA_HUMAN	Prelamin-A/C	+
	5	2.56	22.6	sp|O75083|WDR1_HUMAN	WD repeat-containing protein 1	−
	6	2.49	6.6	sp|O94979|SC31A_HUMAN	Protein transport protein Sec31A	−
	7	2.34	39.9	sp|Q8IVM0|CCD50_HUMAN	Coiled-coil domain-containing protein 50	+
	8	2	10	sp|Q8WV24|PHLA1_HUMAN	Pleckstrin homology-like domain family A member 1	−
	9	1.55	25.5	sp|Q86TI2|DPP9_HUMAN	Dipeptidyl peptidase 9	−
	10	1.55	14.8	sp|Q14764|MVP_HUMAN	Major vault protein	−
	11	0.62	9.1	sp|Q15637|SF01_HUMAN	Splicing factor 1	+
	12	0.49	8.4	sp|P12268|IMDH2_HUMAN	Inosine-5'-monophosphate dehydrogenase 2	−
Ct_E3	1	3.78	34.4	sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3	+
	2	2.38	29.3	sp|Q9P2E9|RRBP1_HUMAN	Ribosome-binding protein 1	Recombinant Ct derived
	3	1.89	14.1	sp|O95817|BAG3_HUMAN	BAG family molecular chaperone regulator 3	–
	4	1.49	19	sp|P27816|MAP4_HUMAN	Microtubule-associated protein 4	−
	5	1.36	12.5	sp|Q86TI2|DPP9_HUMAN	Dipeptidyl peptidase 9	−

Nonspecific proteins identified in mock-transfected HEL cells are excluded from the list. MS/MS spectra of tryptic peptides of SF3b4 are shown in fig. S5.

*Localization of these proteins in the membrane fraction was examined by western blotting with specific antibodies, respectively. +: positive, −:negative

Table S2. Features of 5′ UTR sequences of genes for p180-dependent and independent proteins

Human gene	Kozak consensus sequence*	cDNA sequence from -6 to +4	Expression level after p180 depletion	Number of the motif sequence** in 5' UTR
COL1A1	–	ctagacATGt	down, mRNA shift	4
FN1	–	ctcaacATGc	down, mRNA shift	3
CANX	conserved	gctacgATGg	unchanged	0
MMP2	conserved	cccaccATGg	unchanged	0
TIMP1	conserved	cccaccATGg	unchanged	0

*: A canonical Kozak consensus sequence is "gcc(a/g)ccATGg", which of the − 3 and the + 4 nucleotides are known to be particularly important in efficient translation and are indicated in red.
**: SF3b4-responsive motif sequence (GAG-$(x)_3$-ACA/G/C).

References

(1) Ogawa-Goto K, et al. (2007) p180 is involved in the interaction between the endoplasmic reticulum and microtubules through a novel microtubule-binding and bundling domain. *Mol Biol Cell* 18(10):3741-3751.

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