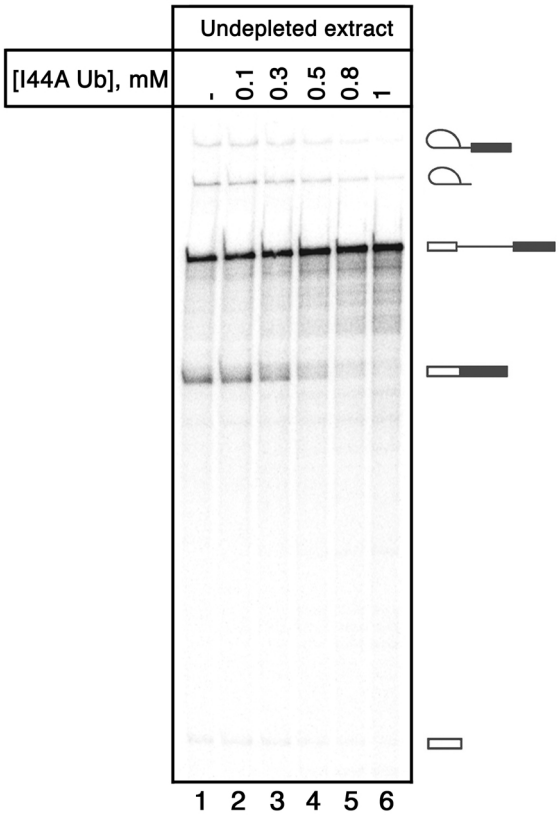
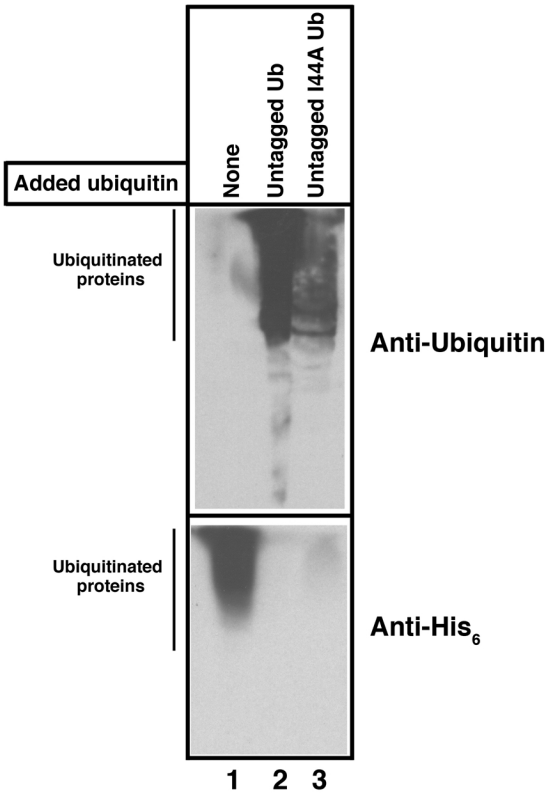
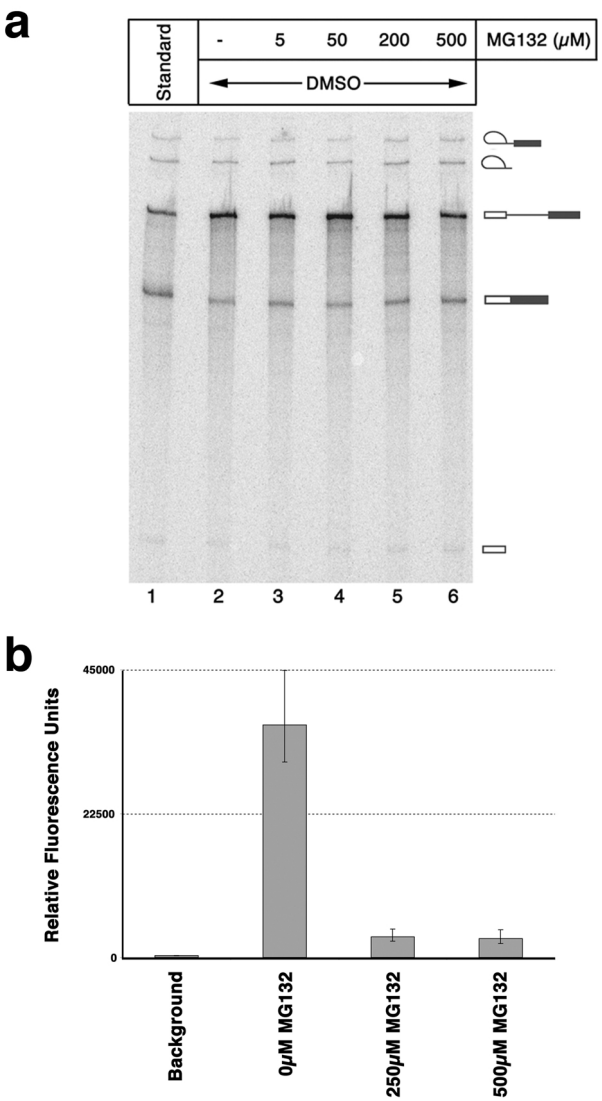
SUPPLEMENTARY DATA



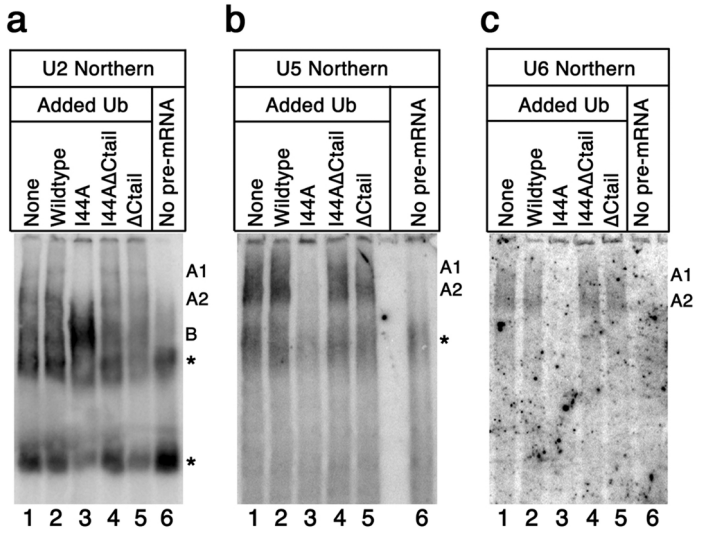
Supplementary Figure 1. A titration of I44A mutant ubiquitin in splicing extract that had not been depleted of ubiquitin. Splicing extracts were prepared from the strain SUB592 (ref. 19), supplemented with I44A ubiquitin at the concentration given at the top of each lane, and then used for *in vitro* splicing reactions. The mobilities of pre-mRNA, splicing intermediates, and splicing products are indicated on the right.



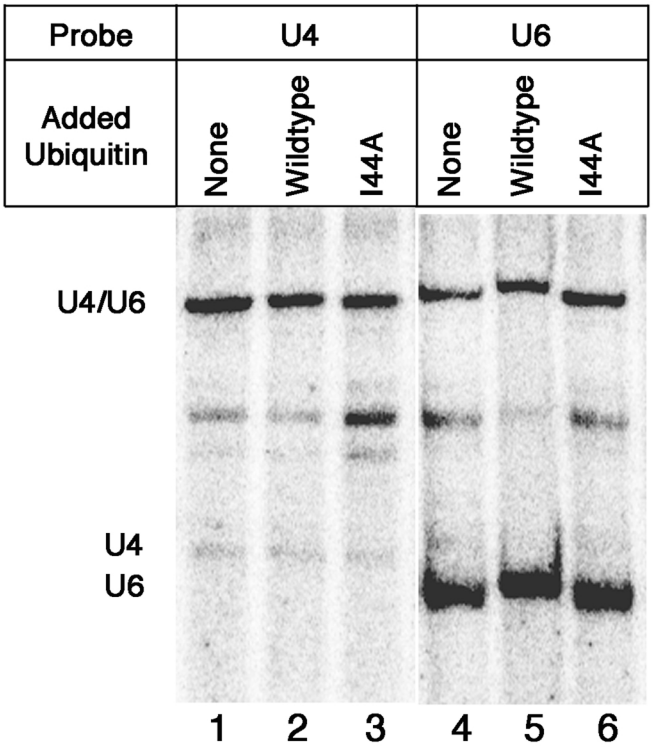
Supplementary Figure 2. I44A mutant ubiquitin is conjugated to proteins in a crude splicing extract. Splicing extract prepared from the strain SUB592 expressing His_6-myc-tagged ubiquitin[19] was supplemented with 1 mM untagged wildtype or I44A ubiquitin. Following a ten-minute incubation, ubiquitin conjugates were detected by immunoblotting with antibodies against ubiquitin (to detect all conjugates) or the His_6 tag (to detect endogenous conjugates), as indicated to the right of each panel. Ubiquitin conjugates are indicated with vertical lines on the left. In the presence of 1 mM exogenous untagged ubiquitin, most of the ubiquitin conjugates lack the His_6 tag, indicating that the exogenous untagged ubiquitin outcompetes the endogenous His_6-tagged ubiquitin. In the anti-ubiquitin blot (upper panel), the proteins conjugated to endogenous ubiquitin (lane 1) are barely detected due to the short exposure time. I44A ubiquitin is conjugated to target proteins, albeit at a lower efficiency than wildtype ubiquitin (compare lanes 2 and 3 of the upper panel).



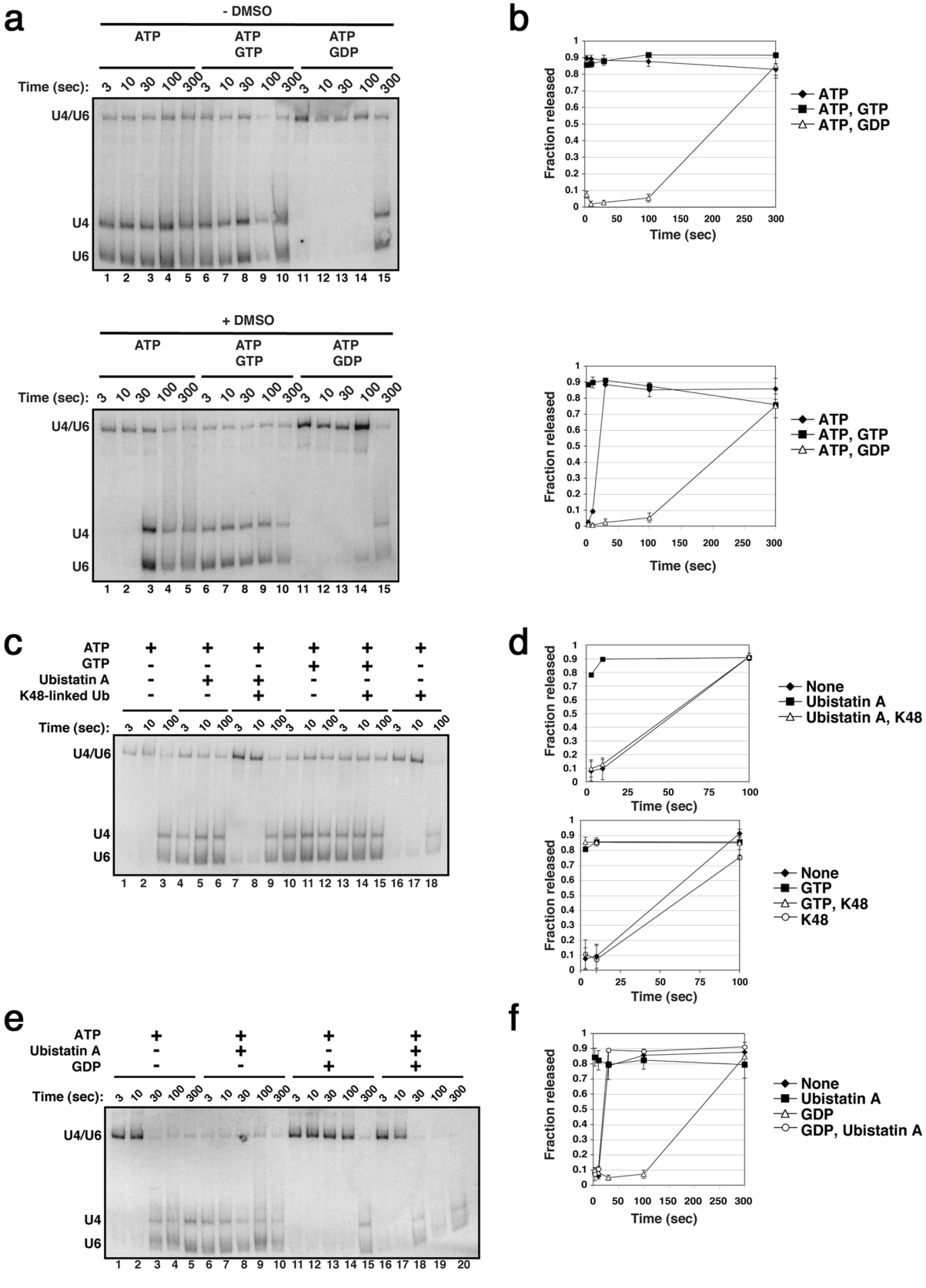
Supplementary Figure 3. MG132 inhibits the proteasome at concentrations that have no inhibitory effect on pre-mRNA splicing. **(a)** *In vitro* splicing reactions were carried out for 30 minutes under standard conditions (lane 1), in the presence of DMSO alone (lane 2), or in the presence of increasing concentrations of the proteasome inhibitor MG132 in DMSO (lanes 3-6). **(b)** A proteasome substrate conjugated to 7-amino-4-methyl-coumarin[4,20] when incubated with splicing extract emits fluorescence upon degradation by the proteasome (0 μM MG132). Proteasome activity is reduced to near-background levels at 250 and 500 μM MG132, which fail to inhibit pre-mRNA splicing as shown in **(a)**. The experiments were done in triplicate, and error bars represent the ranges of the three experiments.



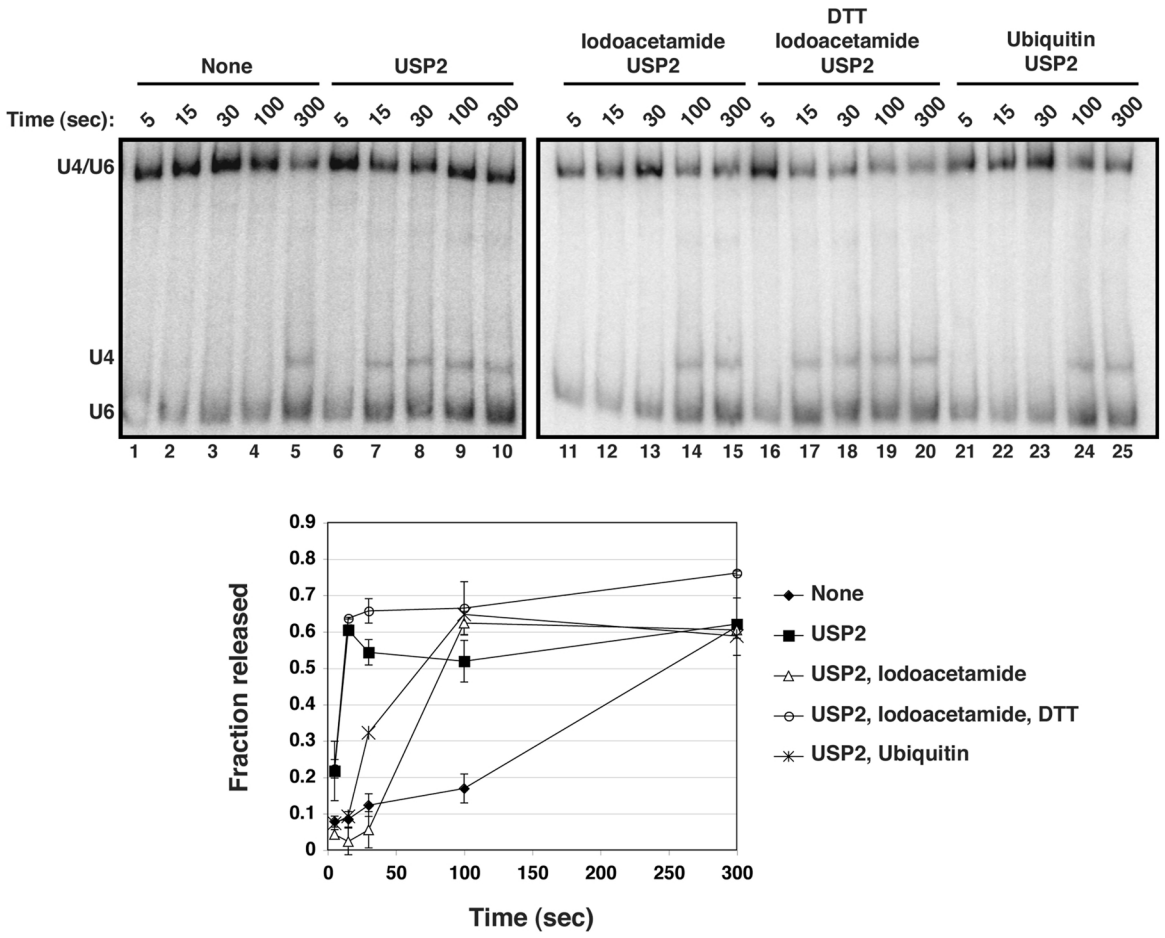
Supplementary Figure 4. Confirmation of pre-spliceosome accumulation in the presence of I44A mutant ubiquitin. Splicing extract was incubated with unlabelled pre-mRNA (lanes 1-5) or no pre-mRNA (lane 6), fractionated by native gel electrophoresis, and analyzed separately by northern analysis with probes specific for the U2 **(a)**, U5 **(b)**, and U6 **(c)** snRNAs. Asterisks indicate endogenous complexes that are present even in the absence of exogenous pre-mRNA.



Supplementary Figure 5. I44A mutant ubiquitin does not induce a U4/U6 di-snRNP defect, as indicated by the presence of base-paired U4 and U6 snRNAs. Following incubation with buffer, 1mM wildtype ubiquitin, or 1mM I44A mutant ubiquitin, splicing extracts were gently deproteinized, annealed to radiolabeled oligonucleotides complementary to either U4 or U6 snRNAs, and analyzed by nondenaturing gel electrophoresis, as described[9]. In all cases we detected a species of the expected mobility that hybridized to both of the U4 and U6 oligonucleotide probes, indicating the presence of annealed U4/U6 snRNAs.


a
- DMSO
ATP
ATP GTP
ATP GDP
Time (sec): 3 10 30 100 300 3 10 30 100 300 3 10 30 100 300
U4/U6
U4
U6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
+ DMSO
ATP
ATP GTP
ATP GDP
Time (sec): 3 10 30 100 300 3 10 30 100 300 3 10 30 100 300
U4/U6
U4
U6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
b
Fraction released
Time (sec)
ATP
ATP, GTP
ATP, GDP
Fraction released
Time (sec)
ATP
ATP, GTP
ATP, GDP
c
ATP + + + + + +
GTP - - - + + -
Ubistatin A - + + - - -
K48-linked Ub - - + - + +
Time (sec): 3 10 100 3 10 100 3 10 100 3 10 100 3 10 100 3 10 100
U4/U6
U4
U6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
d
Fraction released
Time (sec)
None
Ubistatin A
Ubistatin A, K48
Fraction released
Time (sec)
None
GTP
GTP, K48
K48
e
ATP + + + +
Ubistatin A - + - +
GDP - - + +
Time (sec): 3 10 30 100 300 3 10 30 100 300 3 10 30 100 300 3 10 30 100 300
U4/U6
U4
U6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
f
Fraction released
Time (sec)
None
Ubistatin A
GDP
GDP, Ubistatin A

Supplementary Figure 6. Ubistatin A promotes ATP-dependent U4/U6 unwinding in purified U4/U6•U5 triple snRNP particles. **(a)** DMSO, at 0.1%, represses U4/U6 unwinding in purified U4/U6•U5 triple snRNP particles but not in the presence of GTP or GDP. Prp28p-associated triple snRNP particles were TAP-purified and subjected to a previously described U4/U6 unwinding assay[5] in the presence of ATP, with and without guanine nucleotide, as indicated at the top of each lane. Following the reactions, RNA was extracted and fractionated by native gel electrophoresis and analyzed by northern blotting with probes complementary to the U4 and U6 snRNAs. The mobilities of annealed U4/U6 (corresponding to the triple snRNP) and free U4 and U6 (corresponding to the U4 and U6 snRNPs) are indicated on the left. Reactions performed in the absence of DMSO are on top and reactions performed in the presence of DMSO are on the bottom. (**b**) Data from (**a**) and a replicate experiment were imaged with a Phosphorimager, quantitated using ImageQuant and plotted as shown. Again, reactions performed in the absence of DMSO are on top and reactions performed in the presence of DMSO are on the bottom. Error bars represent the range of the two experiments. When the reactions are supplemented with 0.1% DMSO, ATP-dependent disassembly is slowed but in a manner that is counteracted by GTP, suggesting that DMSO promotes the adoption of the GDP-bound state. Consistent with this hypothesis, DMSO does not slow U4/U6 unwinding that is already repressed by GDP. **(c)** U4/U6 unwinding reactions, performed as in **(a)**, show that ubistatin A accelerates unwinding. Reactions included various combinations of ATP, GTP, ubistatin A (20 μM), and K48-linked tetra-ubiquitin chains (60 μM), as indicated at the top of each set of lanes. **(d)** Data from (**c**) and another equivalent experiment were imaged with a Phosphorimager, quantitated using ImageQuant and plotted as shown. Error bars represent the range of the two experiments. **(e)** Reactions performed as in **(a)** show that GDP and ubistatin A (present in this experiment at 50 μM) counteract one another. **(f)** Data from (**e**) and another equivalent experiment were imaged with a Phosphorimager, quantitated using ImageQuant and plotted as shown. Error bars represent the range of the two experiments.



Supplementary Figure 7. The stimulatory effect of the deubiquitinating enzyme USP2 on U4/U6 unwinding is inhibited by free ubiquitin and by the thiol-modifying reagent iodoacetamide. U4/U6 unwinding assays with purified U4/U6•U5 triple snRNP particles were performed, analyzed, and quantitated as described above, except that the particles were pre-treated with the deubiquitinating enzyme USP2 (to remove ubiquitin conjugates) before the addition of ATP (also see **Fig. 5c** and **5d**). All reactions were performed in the presence of GDP. The figure shows control reactions in which USP2 was first treated with either (*i*) iodoacetamide (to chemically modify USP2 cysteine thiols) that was then quenched with DTT (lanes 1-5), (*ii*) iodoacetamide pre-quenched with DTT (lanes 6-10), and (*iii*) 100 mM free ubiquitin (to test for product inhibition of USP2). The reactions with no USP2 (lanes 1-5) and untreated USP2 (lanes 6-10), conducted and analyzed in parallel with the samples in lanes 11-25, are the same as those shown in **Fig. 5c**. In the lower panel, data from the upper panel and a replicate experiment were imaged with a Phosphorimager, quantitated using ImageQuant and plotted as shown. Error bars represent the range of the two experiments.

Supplementary Table 1: Prp8p Peptides Identified by MS

Peptide Sequence	Amino Acids	Xcorr	ΔCN	# of spectra IDed
K.LLENMPHPWEQAK.E	175-187	3.4863	0.4776	1
R.LSTPLRDEVTDK.N	314-325	2.9544	0.4827	1
K.ALNNAIPGGPK.F	341-351	2.5247	0.568	5
R.YIGNLITR.Q	725-732	2.6972	0.4843	1
R.TILQHLSEAWR.C	781-791	2.3186	0.3167	1
K.ADAWVSAAHYNR.E	820-831	2.4444	0.5834	1
R.SFSPIPFPPLTYK.N	894-906	3.2167	0.4879	1
K.ILVLALEDLK.D	911-920	2.8036	0.4468	1
R.VPTSLTSIK.W	1234-1242	1.7111	0.6382	1
R.ISTLFQR.D	1491-1497	2.5736	0.3201	2
K.LWNLNAYR.T	1536-1543	2.2809	0.517	1
K.ANPALYVLR.E	1808-1816	2.4634	0.5116	2
K.LFVDDTNVYR.V	1850-1859	2.3654	0.2942	1
K.TFEGNVATK.A	1865-1873	2.2498	0.4562	1
K.TAEEVSALVR.S	1913-1922	2.4896	0.5449	1
R.LPFSAAMSIDK.L	1963-1973	2.4404	0.5083	1
R.ISSYTAFSR.L	1998-2007	2.3729	0.5444	1
K.SAIANTLLYLR.L	2155-2165	3.2959	0.5434	1

Supplementary Table 1 shows a summary of all peptides identified for Prp8p including the amino acid sequence, amino acid numbers (relative to the N terminus), Xcorr, ΔCN, and number of identified spectra. Xcorr is a cross-correlation score determined by the SEQUEST algorithm that describes the quality of the match between the MS spectra and the highest scoring theoretical spectra[15]. ΔCN is the percent difference in Xcorr between the best and second best match in the database and is an indication of the superiority of the top match relative to other candidate peptides[15].

Supplementary Information

A Role for Ubiquitin in the Spliceosome Assembly Pathway

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SUPPLEMENTARY TEXT

The proteasome inhibitor MG132 does not affect splicing *in vitro*

One of ubiquitin's best-known functions is to be conjugated to a protein target in the form of K48-linked poly-ubiquitin chains[1], which then usually mark the protein for proteolytic destruction by the 26S proteasome. However, many of ubiquitin's regulatory roles are independent of the proteasome[2]. To determine whether ubiquitin's function in splicing reflects a role for proteasome-catalyzed proteolysis, we analyzed pre-mRNA splicing activity *in vitro* in

the presence of the small molecule MG132, which inhibits the proteasome's proteolytic active sites[3]. MG132 had no effect on splicing activity even at 500 μM (**Supplementary Fig. 3a**), which is ~100-fold greater than the concentration necessary to block proteasome activity[3]. As a positive control for the inhibition, a proteasome substrate conjugated to 7-amino-4-methyl-coumarin was used to assay the proteolytic activity of the proteasome by measuring the fluorescence emitted upon degradation of the substrate[4]. MG132 inhibited proteasome activity by ~10-fold at the concentrations that were used in our assay (**Supplementary Fig. 3b**). We conclude that ubiquitin's role in splicing does not require efficient proteolysis by the proteasome.

Pre-spliceosome accumulation induced by I44A ubiquitin

To confirm the identities of the complexes observed in **Figure 2a** and **Figure 2b**, we assembled splicing complexes on unlabeled pre-mRNA and subjected the native gels to Northern blot analysis, since the snRNA composition of each complex is known. In the presence of I44A ubiquitin (but not the wildtype or tailless proteins), a complex accumulates that contains the U2 snRNA (**Supplementary Fig. 4a**), does not contain the U5 or U6 snRNAs (**Supplementary Fig. 4b and 4c**), and comigrates with the pre-mRNA-containing complex (**Fig. 2b**). We therefore conclude that this species is the B complex. Furthermore, slower-migrating U5 and U6 snRNA-containing complexes (such as A2-1, A1, and A2-2) are greatly diminished or abolished in the presence of I44A ubiquitin (**Supplementary Fig. 4b**, lane 3, and **Supplementary Fig. 4c**, lane 3). The U2-containing complex that accumulates in the presence of I44A ubiquitin is not present at significant levels in the absence of added pre-mRNA (**Supplementary Fig. 4a**, compare lanes 3 and 6), confirming its identity as a spliceosome assembly intermediate rather than an endogenous complex.

Ubistatin A accelerates U4/U6 unwinding in purified U4/U6•U5 triple snRNPs

To confirm that U4/U6 unwinding is regulated (in particular, repressed) by ubiquitin, we tested whether ubistatin A derepresses U4/U6 unwinding in triple snRNP particles purified using TAP-tagged Prp28p (ref. 5). Although ubistatin A requires solubilization in DMSO and DMSO slowed the rate of U4/U6 unwinding in the absence of guanine nucleotides (**Supplementary Fig. 5a** and **5b**), we found evidence that DMSO simply favors the repressive GDP state of Snu114p. Specifically, DMSO did not inhibit triple snRNP disassembly that was either repressed by GDP or activated by GTP (**Supplementary Fig. 5a** and **5b**). The inhibition of U4/U6 unwinding by DMSO revealed that GTP can stimulate U4/U6 unwinding, in the absence of GDP, and allowed us to test whether ubistatin A similarly stimulated U4/U6 unwinding.

Consistent with the effects of I44A ubiquitin and USP2, ubistatin A promoted U4/U6 unwinding in the purified particles, indicating that this ubiquitin inhibitor directly activates U4/U6•U5 triple snRNP disassembly. In the presence of ATP and in the absence of ubistatin A, U4/U6 unwinding occurred within 30 s (**Supplementary Fig. 5c** and **5d**). In the presence of ubistatin A, ATP-dependent U4/U6 unwinding occurred within 3 s (**Supplementary Fig. 5c** and **5d**). This enhancement of U4/U6 unwinding was eliminated by a three-fold molar excess of K48-linked tetra-ubiquitin chains, suggesting that U4/U6 unwinding is activated specifically by ubistatin A and not by an impurity (**Supplementary Fig. 5c** and **5d**). Consistent with this interpretation, K48-linked tetra-ubiquitin did not generally repress U4/U6 unwinding, because K48-linked tetra-ubiquitin chains did not (*i*) eliminate the acceleration of U4/U6 unwinding conferred by GTP, or (*ii*) inhibit U4/U6 unwinding in the absence of ubistatin A (**Supplementary Fig. 5c** and **5d**). These data further support a role for ubiquitin in down-regulating U4/U6 unwinding, thereby promoting the stability of the triple snRNP. In addition, the activating effect of ubistatin A on the disassembly of purified U4/U6•U5 particles provides further evidence for a direct requirement for ubiquitin recognition in controlling triple-snRNP dynamics, and suggests strongly that an intrinsic component of the triple snRNP is ubiquitinated.

Intriguingly, ubiquitin and GDP appear to play similar roles in triple snRNP dynamics: both ubiquitin recognition and GDP binding repress U4/U6 unwinding. To determine whether ubiquitin recognition or GDP binding dominates over the other, we added ubistatin A and GDP alone and in combination to purified triple snRNP particles and then determined the time course of U4/U6 unwinding. Whereas ubistatin A accelerated U4/U6 unwinding approximately 10-fold, GDP repressed U4/U6 unwinding approximately 10-fold in the same particles (**Supplementary Fig. 5e** and **5f**). These data indicate that GDP binding can enhance the repressive effect of ubiquitin recognition on U4/U6 unwinding. In addition, in the presence of ubistatin A, the time required for U4/U6 unwinding decreased approximately 10-fold in reactions that were repressed by GDP (**Supplementary Fig. 5e** and **5f**), indicating that ubiquitin recognition can likewise enhance GDP-mediated repression. Conversely, the time required for unwinding in the presence of both ubistatin A and GDP was increased 10-fold compared to ubistatin A alone, indicating that GDP binding can repress U4/U6 unwinding even when ubiquitin recognition is inhibited. Together, these data suggest that ubiquitin recognition and GDP binding each repress U4/U6 unwinding, both independently and in combination.

SUPPLEMENTARY METHODS

Recombinant ubiquitin cloning and purification. The coding region of yeast ubiquitin was amplified by PCR using an N-terminal primer that contained the sequence encoding the His_6-myc tag (MEIHHHHHHAGEQKLISEEDLG). The amplified product was then subcloned into the pET3a vector (Novagen). Plasmids encoding I44A and Ctail mutants were generated using standard mutagenesis procedures and verified by DNA sequencing. The resulting plasmids were transformed into BL21 Codon-Plus competent bacteria (Stratagene). Protein expression was

induced at mid-log phase with 0.5 mM Isopropyl-1-thio-ß-D-galactopyranoside (IPTG) at 37°C for 2 hours. Cells were harvested and resuspended in buffer containing 50 mM sodium phosphate (pH 7.0), 300 mM sodium chloride, and EDTA-free protease inhibitors (Roche) and lysed by sonication at 4°C (24 times for 5 sec each). Cell debris was removed by centrifugation at 20,000g for 40 min at 4°C followed by filtration through a 0.22-μm filter. His_6-myc-tagged ubiquitin was purified by metal affinity chromatography using BD TALON resin (BD Biosciences) according to the manufacturer's instructions, except that the protein was eluted in 10 mM Tris-HCl (pH 8.0) and 150 mM imidazole. The eluate was then loaded onto a HiTrap Q Sepharose resin (GE Healthcare Pharmacia) that had been equilibrated with 10 mM Tris-HCl, pH 8.0 (Buffer A). The protein was eluted using a linear NaCl gradient (0.05 - 0.6 M) in Buffer A, with elution occurring at ~0.19 M sodium chloride. This resulted in >95% pure ubiquitin. The sample was desalted by dialyzing once in Buffer B [0.5 mM potassium phosphate (pH 7.0), and 50 mM NaCl] for 1.5 hours at 4ºC and twice in Buffer C [0.5 mM potassium phosphate (pH 7.0)] for 1.5 hours each at 4ºC. The purified, desalted protein was lyophilized and resuspended in water.

Proteasome assays. Splicing extract was incubated under standard splicing conditions[6] in the presence or absence of increasing amounts of the proteasome inhibitor MG132 (Boston Biochem, Inc.) for 10 minutes, after which DMSO with or without 100 μM of the fluorogenic proteasome substrate Suc-LLVY-AMC (Boston Biochem., Inc.) was added. Following a 20-minute incubation, the reactions were loaded onto a microtiter plate, and fluorescence emission was measured with a fluorimeter (excitation wavelength 370 nm, emission wavelength 465 nm).

Native gels and northern analyses. Spliceosome native gels were done as described[7] with modifications. The gel dimensions were 20 x 16 x 0.08 cm, and electrophoresis was at 200 volts

at 4ºC for 9 hours in buffer containing 40 mM Tris-acetate (pH 7.2) and 1 mM EDTA. For native gel northern analysis, the gel was electroblotted to a HyBond membrane (GE Healthcare) (4ºC, 16 hours, 200 mA) in transfer buffer containing 100 mM Tris base, 50 mM NaOAC, and 5 mM EDTA (pH 7.8). The membrane was then heated between two pieces of Whatman 3 MM paper at 80ºC for 2.5 hours and prehybridized for at least 3 hours in Rapid-Hyb buffer (GE Healthcare) at 65ºC. Hybridization was carried out for 16 hours at 37ºC in 20 mls of the same buffer containing 5 x 10^6 cpm/ml of the labeled DNA probe. The membrane was then washed four times for 10 minutes each in buffer containing 150 mM NaCl, 15 mM sodium citrate, and 0.1% SDS.

We prepared the labeled probes using the NEBlot Kit (New England Biolabs) as described in the manufacturer's instructions. The coding regions of U2, U5 and U6 snRNAs were amplified by PCR, and the product was used as a template for making the labeled DNA probe using α-^{32}P-dCTP (U2 and U6) or α ^{32}P-dATP (U5). Radioisotopes were from MP Biomedical.

Primers. Primers used for reverse transcription assays were as follows: U1RT, 5'-GAATGGAAACGTCAGCAAACAC-3'; U2RT, 5'-CTTAAAAAGTCTCTTCCCGTC-3'; U4RT, 5'-ACCATGAGGAGACGGTCTGG-3'; U5RT, 5'-ACACCCGGATGGTTCTGGTA-3'; U6RT, 5'-AACGAAATAAATCTCTTTGTAAAAC-3'.

Prp8p immunoprecipitation and snRNA primer extension analysis. 30 μL reactions containing 4% extract (corresponding to ~125 μg protein), 60 mM potassium phosphate pH 7.0, 2.5 mM $MgCl_2$, 3% PEG_{8000}, 2 mM ATP, and 1 mM wildtype or mutant ubiquitin or indicated amounts of ubistatin A were incubated at 23ºC-25ºC for 40 minutes. The extracts were prepared from Prp8p^{3HA}-expressing strains [PB592 (this work) or PB2 (ref. 8)]. For reactions that were depleted of ATP, a 10-minute incubation with ubiquitin (wildtype or mutant) or ubistatin A was

done at 23ºC-25ºC in the presence of endogenous ATP, and then 10 mM glucose was added to the reaction and incubated for a further 30 minutes at the same temperature to deplete ATP.

Reactions were quenched with 78 μL of cold Buffer D [20 mM Hepes (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT, and 20% glycerol] and 4.5 μL 0.5 M EDTA, and then added to 15 μL Protein A magnetic beads (New England Biolabs) that had been blocked with BSA and bound to anti-HA antibodies (Sigma). The beads were gently rocked at 4ºC for two hours, and then washed four times with 300-400 μL wash buffer [20 mM HEPES (pH 7.9), 150 mM NaCl, 3 mM $MgCl_2$, 0.5 μM DTT, 15% glycerol, and 0.05% Igepal] for 10 minutes. Beads were then incubated with 1 mg/mL Proteinase K and 0.1% SDS for 15 minutes at 37ºC after which RNA was extracted from the beads with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol.

Reverse transcription analysis was performed on the extracted RNA using the snRNA primers described above. The primers were 5' end labeled using γ-^{32}P-ATP and T4 polynucleotide kinase (New England Biolabs) and then precipitated with ethanol. The extracted RNA from the Prp8p immunoprecipitations was annealed to the primers in annealing buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM EDTA] and 0.2 μM of each primer by heating at 65ºC for 10 minutes followed by slow cooling to 42ºC (at the rate of two minutes per ºC) in a thermocycler. The RNA was then reverse transcribed at 42ºC in extension buffer [50 mM Tris-HCl (pH 8.3 at 42ºC), 5.5 mM $MgCl_2$, 1 mM DTT, and 0.007 μg/μL BSA] using 1.5 U/μL AMV Reverse Transcriptase (Promega). The primer extension products were resolved by 8% denaturing PAGE and analyzed using a PhosphorImager (Molecular Dynamics).

U4/U6 di-snRNP analysis. For the detection of U4/U6 snRNP, standard splicing reactions supplemented with 1 mM ubiquitin (wildtype or I44A) were deproteinized, hybridized to radiolabeled oligonucleotides, and analyzed by nondenaturing gel electrophoresis, as described[9].

Purification of and U4/U6 unwinding in U4/U6•U5 snRNPs. To purify U4/U6•U5 snRNPs from extract in which mutant I44A ubiquitin was conjugated to target proteins, splicing extract was prepared from a strain expressing TAP-tagged Brr2p (yJPS776) as previously described[10]. To conjugate ubiquitin, 1 mM wildtype or mutant I44A ubiquitin (in each case, either full-length or a Ctail mutant) was incubated in 60 μL reactions with 40% extract, 60 mM potassium phosphate (pH 7.0), 2.5 mM $MgCl_2$, 3% PEG, and 2 mM ATP at 25ºC for 10 minutes. To stabilize the U4/U6•U5 snRNP against disassembly, ATP was depleted by adding 10 mM glucose and then incubated for another 30 minutes at 25ºC. Reactions were quenched with 170 μL of 27 mM EDTA in cold buffer D [20 mM Hepes (pH 7.9), 0.2 mM EDTA, 50 mM KCl and 20% glycerol]. The reactions were added to 60 μL IgG sepharose beads (GE Healthcare), gently rocked at 4ºC for three hours, and then washed four times with 1 mL of buffer D. U4/U6 unwinding reactions were assembled and analyzed as previously described[5,11].

Triple-snRNP particles derived from the Prp28p-TAP strain yJPS1004 (ref. 5) were purified and assayed for U4/U6 unwinding as described[5,11]. For reactions containing USP2, before initiating U4/U6 unwinding with ATP, U4/U6•U5 snRNPs were preincubated in splicing buffer at 25°C for 10 minutes with 2.5 M His_6-$USP2_{CD}$ (Boston Biochem) that was or was not inactivated with 2.5 M ubiquitin aldehyde by preincubation at 25°C for 10 minutes. As a control for the specificity of USP2's stimulation of U4/U6 unwinding, U4/U6•U5 triple snRNP disassembly assays with USP2-treated samples were conducted and analyzed as described (see Methods), except that 100 M free ubiquitin (Boston Biochem, Inc.) was added along with the USP2 to determine whether it was subject to product inhibition. Alternatively, the USP2 was pre-treated with 10 mM iodoacetamide at room temperature for 5 minutes to chemically modify cysteine thiols. The iodoacetamide was quenched with 25 mM DTT, and the reacted USP2 was

then used in the assay. We also performed a pre-quench control reaction in which the DTT and iodoacetamide were mixed before the addition of USP2.

For reactions containing ubistatin A, U4/U6•U5 snRNPs in splicing buffer were preincubated with ubistatin A for 10 minutes on ice before initiating reactions with ATP. K48-linked tetra-ubiquitin chains were preincubated with ubistatin A for 10 min at room temperature followed by an additional 10 min incubation with U4/U6•U5 snRNPs in splicing buffer on ice prior to initiating the reactions with ATP.

Mass spectrometric identification of ubiquitin conjugates. To identify ubiquitin conjugates in the U4/U6•U5 triple snRNP, particles derived from the Brr2p-TAP strains yJPS1274 and yJPS1275 (expressing untagged and His_6-tagged ubiquitin, respectively) were affinity-purified with IgG-sepharose as previously described[5], starting with 12 L of culture. Following TEV elution in 500 µL of TEV cleavage buffer (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 50 mM KCl, 20% [v/v] glycerol), the eluted snRNPs were brought up in 5 mL binding buffer (50 mM sodium phosphate buffer [pH 8.0], 8 M urea, 300 mM NaCl, 0.5% Triton x-100) and 500 µL Ni^{2+}-NTA agarose resin (Qiagen) and allowed to bind overnight at 25°C. The resin was washed twice with 5 mL binding buffer and three times with 5 mL wash buffer (50 mM sodium phosphate buffer [pH 6.3], 8 M urea, 300 mM NaCl, 0.5% Triton X-100). The bound material was eluted four times with 500 µL elution buffer (50 mM sodium phosphate buffer [pH 4.3], 8 M urea, 300 mM NaCl, 0.5% Triton X-100). The eluted fractions were pooled and precipitated with trichloroacetic acid (TCA). TCA was added to a final concentration of 20% and incubated on ice for 30 minutes. After centrifugation at 10,000 g for 15 minutes, the pellets were washed with 500 µL ice-cold acetone, re-centrifuged, and washed again. Each pellet was resuspended in 50 µL of digestion buffer (50 mM Tris [pH 8.5], 8 M urea). To reduce cystines present in the samples, Tris-carboxyethylphosphine (TCEP) was added to a final concentration of 5 mM and incubated

for 30 min at 25°C. Free cysteine residues were then carbamidomethylated by the addition of iodoacetamide (10 mM final) followed by incubation for 30 min at 25°C. One μL of Lys-C protease (0.1 μg/μL) was added to each sample and incubated for 4 hrs at 37°C. After Lys-C digestion, the samples were diluted to a concentration of 2M urea by the addition of three volumes of 100 mM Tris [pH 8.5], supplemented with $CaCl_2$ (1 mM final), and digested by the addition of 2 μL of trypsin (0.4 μg/μL) followed by incubation for 4 hrs at 37°C. Digested samples were acidified by the addition of formic acid to a final concentration of 5% and then analyzed by MudPIT or 2D-LC-MS/MS essentially as previously described[12-14]. Briefly, each sample was pressure-loaded onto a 100 μM inner diameter fused silica capillary column with a 5 μM pulled electrospray tip that had been previously packed with 8 cm of Luna C18 3 μM reverse phase particles (Phenomenex) proximal to the tip followed by 3 cm of Luna strong cation-exchange (SCX) resin (Phenomenex) and 3 cm of Luna C18 5 μm reverse phase particles. After loading and equilibration, the capillary column was placed in-line with the mass spectrometer and fractionated using a modified 7-step separation strategy. Peptides were eluted directly into a LTQ-Orbitrap mass spectrometer and data was collected according to ref. 14. Analysis of MS/MS spectra was performed using the SEQUEST algorithm and DTASelect2 (refs. 15 and 16). The criteria used for a positive protein identification in these experiments was the identification of at least two unique fully-tryptic peptides with an estimated false positive rate of less than 5% at the spectral level as determined using a decoy database strategy[17]. All MS data is available upon request.

To increase the yield of recovered protein by avoiding any potential samples losses that might have occurred during TCA precipitation step, we repeated the analysis but trypsinized the sample directly on the Ni^{2+}-NTA agarose resin. Binding to the resin was performed as described above except that the Ni^{2+}-NTA agarose resin was reduced to 50 μL. The resin was washed two times with 1 mL binding buffer and two times with 1 mL wash buffer (50 mM Tris [pH 8.5], 8 M

urea). Reduction, carbamidomethylation, and sequential digestion of the sample were performed as above except the samples were agitated throughout the procedure to prevent the resin from settling in the bottom of the tube. Samples were analyzed by MudPIT as described above.

Western blot detection of Prp8p/ubiquitin conjugates. To confirm the presence of ubiquitinated Prp8p in the U4/U6•U5 triple snRNP, the particles were purified as described for the mass spectrometric analysis above, up to and including washing the Ni^{2+}-NTA resin with wash buffer. To elute the samples from the resin, the samples were boiled for 5 min in SDS loading buffer. The samples were separated on a 4-20% protein gel (Pierce) and electroblotted onto a nitrocellulose membrane. The blot was probed for Prp8p with anti-8.6 antibodies[18] and developed using Rodeo Sensitive Detection Kit (US Biochemicals).

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