**Figure S1. Analysis of variant U1 snRNA expression by Northern blotting.** To confirm processing of the U1 snRNA to mature length, total RNA from HeLa cells expressing U1-5a variants carrying single and double SL3/4 mutations was probed with 32P-U126-46R oligonucleotide to detect both endogenous and transiently expressed U1 snRNAs. U2 snRNA was detected using 32P-U2114-135R and serves as a loading control.

**Figure S2. U1 snRNA carrying single and double mutations exhibit nuclear localization and associate with U1-70k.** Detergent based hypotonic lysis buffer efficiently fractionates HeLa cells for isolation of nuclear and cytosolic components. (A) Northern blot of RNA isolated from nuclear and cytoplasmic fractions was probed with U1, U2, and 5S rRNA specific oligonucleotides (Supplementary Data File Table S5). The average RNA levels (± s.d., n = 3) in subcellular fractions calculated as a percentage of total RNA is graphed. (B) Western analysis of nuclear and cytoplasmic fractions using antibodies against U1-70K, SF3A1, SF3B1, and α-Tubulin. The average protein level (± s.d., n = 3) in subcellular fractions expressed as a percentage of total protein is graphed. (C) Northern blotting of RNA from total cell lysate (T), and nuclear (N) and cytoplasmic (C) fractionations from HeLa cells expressing U1-5a variants carrying wildtype SL3/SL4, single, or double mutations. Northern blots were probed with 32P-labeled oligonucleotides specific to U1 and U2 snRNAs, 5S rRNA, and the SL4-M10r mutation (Supplementary Data File Table S5). (D) Immunoblot of nuclear and cytoplasmic fractions using anti-SF3A1 and anti-α-Tubulin antibodies.

**Figure S3. U1 snRNAs carrying single and double mutations exhibit normal processing.** (A) Anti-U1-70k antibody efficiently immunoprecipitates U1 snRNP from HeLa nuclear extracts. Protein from input (I), flow through (FT), and immunoprecipitated (IP) complexes were collected during IP of HeLa nuclear extracts with anti-U1-70k antibody and analyzed by Western blotting using the same U1-70k antibody. The positions of U1-70k and the IgG heavy chain are indicated. (B) Northern blotting analysis of RNA extracted from total cell lysate (T), nuclear fractions used as input (I), and anti-U1-70k antibody IP complexes (IP). Lysates and nuclear fractions were derived from HeLa cells expressing single SL4-M10r or double SL3/4 mutations. A slight change in the mobility of the U1-5a M10r mutant snRNA was observed in the immunoprecipitated RNA fraction (Lane 21; \*). This reduced mobility band was not seen in total or input RNA from the same lysate, and may, therefore, be occurring as a result of processing and handling of lysate during immunoprecipitation.

**Figure S4. U1-SL3 interacts with RNA helicase UAP56.** (A) UV-crosslinking analysis of wildtype and mutant 32P-U1-SL3 RNAs in the presence of increasing NaCl concentration (0 - 1.0 M NaCl). Interacting proteins were UV crosslinked and analyzed by SDS-PAGE. (B) snRNA analysis of the complexes that bind to biotinylated U1-SL4-WT, U1-SL3-WT, and U1-SL3-M1g RNAs in HeLa nuclear extract. Total RNA was extracted from complexes, separated by urea-PAGE and visualized by GelRed total nucleic acid staining. Positions of the spliceosomal snRNAs are indicated.

**Figure S5. GST-UAP56 specifically binds U1-SL3, but not U1-SL4, *in vitro*.** (A) Coomassie stained SDS-PAGE gel of purified GST, GST- SF3A1-UBL, and GST-UAP56 proteins that were used in RNA binding experiments. (B) EMSAs monitoring binding of Cy5-labeled U1-SL4-WT RNAs (50 nM) to GST or GST-UAP56 (2.0 μM) in the presence of either ATP (lanes 1-3) or ATP-γ-S (lanes 4-6). (C) EMSA monitoring binding of Cy5-labeled U1-SL3 (lanes 1-7) or U1-SL4 (lanes 8-14) RNAs (10 nM) to GST-UAP56 (0.125, 0.25, 0.5, 1.0, 2.0, 8.0 μM) was performed in the presence of ATP-γ-S. Dose-response curves were generated from EMSA images by plotting the average percent of bound U1-SL3 and U1-SL4 RNA (± s.d., n = 3) versus GST-UAP56 protein concentration and is displayed to the right of the gel image (n = 3). (D) EMSAs monitoring binding of Cy5-labeled U1-SL3-WT RNAs (50 nM) to GST (2.0 μM) or GST-UAP56 (0.25, 0.5, 1.0, and 2.0 μM) in the presence of either ATP (lanes 1-6) or ATP-γ-S (lanes 7-12). (E) EMSA for determining NTP specificity of UAP56 was performed with 50 nM Cy5-U1-SL3 and 2.0 μM GST or GST-UAP56 in the presence of ATP-γ-S, ATP, UTP, GTP, or CTP. (F) Urea-PAGE analysis of RNA present in input (I) and U1 affinity purified (AP) complexes in the absence and presence of ATP-γ-S or ATP. (G) Urea-PAGE analysis of RNA present in input (I) and U2 AP complexes in the absence and presence of ATP-γ-S or ATP. RNA in the purified complexes were detected using GelRed total nucleic acid staining of urea-PAGE gels.

**Figure S6. Combination of UAP56 or URH49 knockdown with U1-SL4 M10r mutation has mild synergistic effect.** (A) Primer extension analysis of Dup51p reporter transcripts after complementation with U1-5a variants and treatment with control siNT, siUAP56 or siURH49. The average percentage of the full-length product (± s.d., n = 3) is graphed below (siUAP56 n = 3, siURH49 n = 2; \* = *p* < 0.05, \*\* = *p* < 0.01). Statistical comparisons were performed for each U1-5a snRNA tested under the siNT versus siRNA treatment conditions. (B) Table reporting results of the analysis for synergy performed for U1 snRNAs carrying WT or single SL3 or SL4 mutations under UAP56/URH49 knockdown conditions. The coefficient is the average difference between the expected and observed values for U1 activity, a positive coefficient indicates synergy however the UAP56/URH49 knock down in combination with SL4-M10r mutation did not meet our stringent criteria (difference ≥ 0.1 and *p* < 0.01).

**Figure S7.** **U1-SL3 promotes U1-SL4/SF3A1 interaction in an ATP-dependent manner.** HeLa nuclear extracts containing 32P-U1-SL4 were incubated in the absence of ATP (A) or in the presence of ATP-γ-S (B). The complexes were UV crosslinked, treated with RNase T1, and then analyzed by SDS-PAGE. To determine the effect of free U1-SL3 and U1-SL4, the reactions were preincubated with 0.625, 1.25, 2.5, and 5.0 μM of the indicated cold competitor stem-loop RNAs prior to addition of 32P-U1-SL4. (C) *In vitro* splicing of uniformly 32P-labeled AdML pre-mRNA in the absence of stem-loop RNA or in the presence of 0.625, 1.25, 2.5, and 5.0 µM mutant U1-SL3 M1g RNA. Splicing intermediates and products are depicted. Fold change in splicing activity is the mRNA/pre-mRNA ratio calculated relative to the no SL control. Statistical analysis compared activity in the presence of U1-SL3 to the no SL control (± s.d., n = 2; \* = *p* < 0.05, \*\* = *p* < 0.01). (D) Primer extension analysis with oligonucleotide 32P-U17-26R (Supplementary Data File Table S5), showing expression of the U1-5a harboring wildtype snRNA or snRNA carrying tandem SL4 (SL4/SL4), tandem SL3 (SL3/SL3), or swapped SL3 and SL4 (SL4/SL3).

**Supplementary Data File.** Table S1: NSAF analysis for MS data. Table S2: Spliceosomal proteins identified in the wildtype and mutant U1-SL3 complex. Table S3: List of peptides for proteins identified by MS analysis of the U1-SL3-WT complex. Table S4: List of peptides for proteins identified by MS analysis of the U1-SL3-M1g mutant complex. Table S5: Sequences of oligonucleotides and small interfering RNAs used in this study. Table S6: Sequences of primers used for creating SL3 and SL4 mutations in the U1-5a snRNA.