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A dual reporter approach to quantify defects in mRNA processing

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Abstract

Splicing and nuclear export are vital components of eukaryotic gene expression. Defects in splicing due to cis-mutations are known to cause a number of human diseases. Here we present a dual reporter system that can be utilized to look at splicing or export deficiencies resulting from an insufficiency in components of the co-transcriptional machinery. The constructs utilize a bidirectional promoter to co-express a test and a control reporter. In the splicing construct, maximal expression of the test reporter is dependent on efficient splicing and splicing related nuclear export, while the control reporter is an intronless cDNA expression cassette. The dual reporters allow for a robust ratiometric output that is independent of cell number or transfection efficiency. Therefore, our construct is internally controlled and amenable to high throughput analysis. As a counter-screen we have a non-splicing control construct in which neither reporter bears an intron. We demonstrate the sensitivity of our construct to defects in nuclear export by depleting UAP56 and NXF1, essential components of the co-transcriptional machinery.

Keywords

mRNA; splicing; nuclear export; UAP56; NXF1; dual reporter assay

Introduction

Eukaryotic gene expression is a complex process involving several pre-mRNA processing steps that culminate in nuclear export and translation of the mature mRNA. As it emerges from RNA polymerase II the nascent pre-mRNA interacts with a large number of proteins that determine whether it is spliced and exported from the nucleus. Multiple lines of evidence point to a co-transcriptional assembly of ribonucleoprotein particles (mRNPs), in which transcription, splicing and export factors act in concert [1;2]. The machinery involved in these processes is largely conserved from yeasts to metazoans. In yeast, the THO/TREX protein complex aids in transcription elongation and couples transcription to mRNA export [3;4]. THO is a four-protein complex (Hpr1, Tho1, Mft1 and Thp2) that associates with mRNA export proteins Sub2 and Yra1 within the larger TREX (transcription/export) complex [5]. Mutations in the THO/TREX constituent proteins can lead to a hyper-recombination phenotype and impaired gene expression [6;7]. As the model for co-transcriptional events is better formulated, additional proteins are being identified to be important for efficient mRNP assembly. A mutation or

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insufficiency in one or more of these proteins would likely cause a breakdown in the processing of mRNA and reduced gene expression in the subset of genes using that export pathway.

A number of human diseases result from splicing abnormalities [20;21]. Many of the studies analyzing these disorders have looked at alternative splicing and exon skipping, as a result of sequence variations in splice donor and acceptor sites in individual genes [22;23;24]. At present the most common method to look for splicing deficiencies involves RNA isolation and RT-PCR, which can be time consuming. Differing efficiencies of amplification in RT-PCR can also lead to errors in calculating the levels of spliced and unspliced products. Microinjection of radiolabeled RNA or plasmid DNA into nuclei has been used to demonstrate the importance of splicing for nuclear export [25;26]. Rodrigues et al evaluated the role of REF/Aly, an export factor, in nuclear export of mRNA by microinjecting REF anti-sera into *Xenopus* oocyte nuclei along with labeled mRNAs. Nuclear injections are usually followed by analysis using FISH [25;26] or resolution of nuclear and cytoplasmic fractions on denaturing polyacrylamide gels followed by autoradiography [27]. These techniques have proven to be invaluable for the understanding of co-transcriptional processes. However, the protocols mentioned above can be technically challenging and laborious. A fast and easy transfection-based reporter assay would be useful in providing a quick screen of splicing abnormalities, which can then be further characterized by other protocols.

Here we describe a reporter system designed to provide a sensitive assay for breakdowns resulting from deficits in components of the co-transcriptional machinery. This system utilizes a bidirectional tetracycline inducible promoter and co-expresses two luciferase reporters, one of which contains an intron. Optimal expression of the test reporter is dependent on splicing and splicing-related export similar to a native gene, while that of the control reporter is not. To probe the sensitivity of our construct to defects in mRNA processing we chose to knockdown proteins that have a well-characterized function in splicing and export. We demonstrate that the depletion of UAP56 and NXF1 leads to a reduction in expression of our splicing reporter compared to an intronless control.

Methods

1. Plasmids and cloning

An intronless construct (BI-16) with a bidirectional promoter [28] was our control and served as the base for the minigene construct (SPLCX) (Fig. 1A). The promoter consists of two asymmetric lengths of the Invitrogen Pcmv (2 $\times$ -TetO₂) promoter joined tail to tail. This promoter has been previously characterized in detail by our lab [28]. Human *FXN* exon 1 and 2 sequences were PCR amplified from genomic DNA. Using the numbering on chr9 March 2006, the *FXN* exon 1 splice donor is at (70840683) followed by a consensus GTAAGT. Nested PCR was used to insert a BglII restriction site followed by an ATG at frataxin amino acid 42 (70840642) and a SpeI site after 100 bp of the intron (70840783) to make frag1. The *FXN* splice acceptor is at (70851121) preceded by 16/20 consensus in the -20 region. Nested PCR was used to insert an XbaI site 5' to the last 90 bp of intron I (70851031) and after 60 bp of exon 2 (70851181), an AflIII site (creating an in-frame TAA) and BglII and HindIII sites to create frag2. PCR was used to add BamHI, XmaI and XhoI to one side of a 361 bp fragment of the CAT gene from pSV2CAT (4969-4608 bp Genbank M77788) and XbaI was added to the other side to make frag3. Frag1 and pREX [29] were cut with BglII and SpeI and ligated. The resulting plasmid was cut with SpeI and BamHI and ligated to XbaI and BamHI cut frag2. Frag3 and the plasmid with fragments 1 and 2 were cut with HindIII and frag4 was inserted. The final intermediate plasmid was cut by BglII, which flanks 1, 2, 3 and 4, and the fragment was ligated into BamHI cut pcDN/FRT/FL-/TETBI/RL [28] to create the SPLCX construct lacking the translational re-initiation spacer. This was cut with NcoI (partial) and AflIII and the

75 bp re-initiation spacer from GAP-43 transgene [30] was cut with AflIII and NcoI then ligated in to complete the SPLCX reporter construct.

2. Cell culture, transfections, and cell lines

Transient transfections using 250 ng of our reporter plasmids were carried out using Lipofectamine 2000TM (Invitrogen) as per the manufacturers protocol. For establishing stable cell lines, commercially available HEK 293 Flp-InTM T-RExTM cells (Invitrogen) were co-transfected with 100 ng of the vector carrying our reporter construct and 900 ng of pOG44 using Lipofectamine 2000TM (Invitrogen). HEK 293 Flp-InTM T-RExTM cells have a single stably integrated Flp recombinase target (FRT) site and constitutively express Tet repressor. These cells are designed for use with Invitrogen's Flp-InTM system. Cells transfected to make stable cell lines were selected with hygromycin and blasticidin, 75 µg/ml and 15 µg/ml respectively. Individual colonies were picked and expanded under antibiotic selection.

3. Dual luciferase assay

The dual luciferase (DLR) assay was performed using a DLR assay kit (Promega) according to the manufacturer's directions. Briefly, cells were lysed with Passive lysis buffer (Promega), and 20 µl of lysate was assayed in white 96-well luminescence plates (Dynex). Luciferase expression was measured using a Veritas dual injector plate reader luminometer (Turner Biosystems). Lysates obtained from a doxycycline-induced control cell line [28] were used as a positive control. Ratios were calculated for expression of sea pansy luciferase (*Renilla reniformis*) to firefly luciferase (*Photinus pyralis*) (hRLUC/FLUC). All ratios were normalized to the average ratios of the four positive controls.

4. Nuclear isolation

Nuclear isolation was performed using the protocol described by Greenberg et al [31]. Cells were scraped in cold PBS and pelleted at $500 \times g$ at 4 °C for 5 minutes. The cell pellet was resuspended in NP-40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) NP-40), and incubated on ice for 5 minutes. Nuclei were centrifuged at $500 \times g$, and the supernatant containing the cytoplasmic fraction was saved.

5. shRNA-mediated depletion

For depleting UAP56, NXF1 and ASF we used the pLKO.1 vector system from Open Biosystems, which confers puromycin resistance and drives shRNA expression from a human U6 promoter. The shRNA sequences for UAP56, NXF1 and ASF correspond to TRCN0000074384, TRCN0000007581 and TRCN0000001094 respectively, on the Open Biosystems shRNA database. HEK293 cells were transfected with either the empty pLKO.1 vector or the vector carrying the protein-specific shRNA construct, and selected with 1 µg/ml puromycin.

6. Immunoblotting

Cells were lysed in 2× Laemmli buffer (2% SDS, 20% Glycerol, 100 mM Tris pH 6.8 and 125 mM DTT). 100 µg of whole cell lysate was resolved on an 8% resolving SDS polyacrylamide gel (37.5:1). Proteins were transferred to Immobilon-P membrane (Millipore), using a semi-dry transfer apparatus (Biorad). The membrane was blocked in 20% evaporated carnation milk in PBS for 1 hour at room temperature, and then incubated with either mouse anti-human UAP56 antibody (1:1000 dilution) or mouse anti-actin antibody (1:5000) overnight at 4°C. A horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce) was then used (1:10000 dilution) followed by detection using the ECL advance chemiluminescence kit (Amersham). A Kodak Gel Logic 440 Imaging system was used for imaging, and band intensities were analyzed using the Kodak Molecular Imaging Software (Version 4.0.5f7). The

statistical significance in the difference between the mean band intensities of various samples was determined using an unpaired Student t-test, assuming unequal variance in the data groups.

7. cDNA synthesis and Real time PCR

RNA was extracted from whole cells, nuclei, or cytoplasmic samples using TRI-Reagent (Molecular Research Center, Inc.). First strand cDNA synthesis was carried out using the Sidestep II QPCR cDNA synthesis kit (Stratagene), as per the manufacturers protocol. Quantitative real time PCR reactions were set up using the iQ SYBR Green supemix (Biorad). Primer sets for FLUC and hRLUC were obtained from IDT (Coralville, IA), and used at a final concentration of 200 nM. Primer set for PCR to demonstrate splicing: FR1XF1, tgggaagttcttctgaggt; RL5-80F, ggagtcacgacgttcatt. Primer sets for real time PCR on nuclear and cytosolic sample: FLUC sense, aagattcaaagtgcgctgctggtg; FLUC antisense, ttgcctgatacctggcagatggaa; hRLUC sense, aatggctcatatcgctcctctggat; hRLUC antisense, tggacgatcgccttgatctgtct.

Real time analysis was conducted using a MX3000P sequence detection system from Stratagene. Standard curves for each primer set (FLUC and hRLUC) were made using serial dilutions of the pBI-16-FTH plasmid, which expresses both reporters [32]. These standard curves were then used in the MX3000P software (version 2.0) to calculate the copy number for FLUC and hRLUC. The cycling conditions were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 30 seconds. Analysis was done using the MX3000P software (version 2.0).

8. Northern blot

For FLUC and hRLUC mRNA detection, cells were electroporated with 50 µg of plasmid at 250 V, 1000 µF using a Gene Pulser apparatus (Bio-Rad), and plated overnight in media containing 1 µg/ml doxycycline. For all northern blots RNA was isolated using TRI-Reagent (Molecular Research Center, Inc.). 20 µg of RNA was resolved on a denaturing 1% MOPS/formaldehyde-agarose gel, and transferred to Hybond N+ membrane (Millipore). The RNA was UV-crosslinked to the membrane for 1 minute, and then the membrane was pre-hybridized in ULTRAhybe™ (Ambion) at 65°C for an hour. RNA probes radiolabeled with [α -³²P] - UTP (MP Biomedicals) were synthesized using *in vitro* transcription with T7 RNA polymerase from HindIII linearized plasmids carrying FLUC, hRLUC, NXF1, ASF or GAPDH in anti-sense orientation to a T7 promoter. The pre-hybridized membrane was incubated with probe overnight at 65°C. The membrane was washed, air dried and exposed to a phosphorscreen overnight. A Typhoon phosphorimager was used to obtain an image, followed by analysis of band intensities with the Kodak Molecular Imaging Software (Version 4.0.5f7). The statistical significance in the difference between the mean band intensities of various samples was determined using an unpaired Student t-test, assuming unequal variance in the data groups. For measuring NXF1 and ASF knockdowns, their respective mRNA band intensities were normalized to that of GAPDH and compared to similarly normalized values obtained from wildtype cells.

Results and Discussion

A bidirectional construct demonstrates enhanced nuclear export of a processed reporter

To study the influence of co-transcriptional processing on gene expression, we designed a pair of bidirectional reporter constructs: an intron-bearing splicing and export construct (SPLCX) and a simple intronless cDNA control construct (BI-16) [28] (Fig. 1A). The constructs co-express firefly luciferase (FLUC) and humanized renilla luciferase (hRLUC) reporters under the control of a bidirectional tetracycline inducible promoter. SPLCX has an intron with splice donor and acceptor sites cloned upstream of the hRLUC reporter (Fig. 1A). In order for hRLUC

to be effectively expressed from SPLCX, successful splicing of the intron and export of the hRLUC mRNA is required. FLUC reporter expression does not require splicing and serves as an internal control. Expression of both reporters (FLUC and hRLUC) from BI-16 is independent of splicing. To confirm successful splicing in the SPLCX construct, we reverse transcribed RNA from transiently transfected cells and analyzed hRLUC splicing by PCR. The RT-PCR showed a spliced product at the expected size and did not detect any unspliced product (Fig. 1B). The spliced product was gel purified and sequenced to verify correct splicing (Fig. 1C).

To determine if there was a differential sub-cellular distribution of the spliced and unspliced reporter mRNAs in SPLCX, we fractionated cell extracts into nuclear and cytoplasmic fractions and measured the levels of hRLUC and FLUC mRNA. For SPLCX, real-time PCR indicated approximately 3-fold more hRLUC mRNA in the cytoplasm as compared to the nucleus (Fig. 2). In cells carrying the intronless control construct BI-16, hRLUC mRNA was not preferentially exported to the cytoplasm. FLUC mRNA levels for both constructs were similar in the nucleus and cytoplasm (Fig. 2).

Splicing has been shown to recruit the TREX complex to mRNA, which in turn mediates active export of the message via the nuclear pore complex [10]. In accordance with this our spliced hRLUC reporter shows a significant advantage in nuclear export over the unspliced controls ($p < 0.05$). This increased export of hRLUC most likely is the result of the SPLCX spliced message being cotranscriptionally processed like a native pre-mRNA molecule.

Enhanced nuclear export corresponds to increased hRLUC expression

All of our constructs contain an internal control in the form of the second reporter (FLUC). The expression of the test reporter (hRLUC) can be normalized to that of the control reporter (FLUC). This ratiometric output makes the assay independent of cell number and transfection efficiency and so our system is ideal for high-throughput screening. Luciferase expression from SPLCX and BI-16 was analyzed after transient transfections into HEK 293 cells (Fig. 3). Transient transfections demonstrated an approximately 2.4 fold increase in expression of the hRLUC reporter from SPLCX when compared to BI-16. We also established stable cell lines with single copy integration of our constructs into the same unique chromosomal location using the Invitrogen Flp-In™ system to determine if a single copy reporter system would yield different results. Luciferase expression from the stable cell lines showed an approximately 2.8 fold enhanced hRLUC expression over an unprocessed hRLUC (Fig. 3). Luciferase expression from our constructs, as for any gene, is dependent on mRNA translation, which in turn relies on efficient nucleo-cytoplasmic export of the message via the nuclear pore. Splicing has been shown to play a key role in the recruitment of the export machinery to metazoan mRNA, and is therefore vital for optimal expression.

The spliced hRLUC reporter is sensitive to depletion of co-transcriptional proteins

We have demonstrated that the enhanced hRLUC expression from the SPLCX construct corresponded to an increased nuclear export of its message. Since splicing and export are intricately linked we wanted to investigate how sensitive our reporter system would be to defects in co-transcriptional processing. The human orthologs of the TREX complex mentioned previously, Sub2 and Yra1 (UAP56 and REF/Aly, respectively) are recruited to the pre-mRNA during a late step of splicing [10;11]. UAP56 is an ATP-dependant RNA helicase belonging to the DEAD/H-box family that plays an important role in spliceosome assembly [12;13]. Recent reports suggest that UAP56 acts as an intermediate between splicing and export by recruiting REF/Aly to the mRNA [17]. REF/Aly is an export factor that acts as an adaptor by interacting with NXF1/TAP, which in turn promotes export of mRNA through nuclear pore complexes [14;15]. We used shRNA to deplete UAP56 and used western blots to demonstrate

an approximately 50% knockdown of protein expression (Fig. 4A). We transiently transfected the UAP56 depleted cells with SPLCX or the control BI-16 to analyze hRLUC expression (Fig. 4B). A substantial decrease in hRLUC/FLUC ratios from SPLCX was seen in cells with UAP56 knocked down when compared to control cells with normal UAP56 levels ($p<0.05$) (Fig. 4B). BI-16 was not sensitive to UAP56 depletion and did not show a significant decrease in hRLUC/FLUC ratios.

To determine the cause of the altered expression, nuclear and cytoplasmic fractions were prepared from UAP56 depleted cells transiently transfected with either SPLCX or the BI-16 construct. The sub-cellular distribution of the hRLUC message was analyzed by real-time RT-PCR. Lowered levels of cytoplasmic hRLUC mRNA transcribed from SPLCX were observed in cells with reduced levels of UAP56 (compare Fig. 2 and Fig. 4C). BI-16 did not demonstrate a change in hRLUC mRNA distribution in response to UAP56 knockdown (Fig. 4C). Northern blot analysis of FLUC and hRLUC mRNA was carried out on wildtype and UAP56 knockdown cells transfected with BI-16 or SPLCX (Fig. 4D). A percentage of unspliced hRLUC mRNA was observed in SPLCX transfected cells. The UAP56 depleted cells electroporated better than the wildtype cells, and showed higher net levels of hRLUC and FLUC mRNA. Densitometric analysis of the bands showed that the ratio of unspliced to spliced hRLUC mRNA did not show a significant increase in the UAP56 knockdown cells as compared to wildtype cells transfected with SPLCX (Fig. 4E). This indicates that the depletion of UAP56 to 50% of normal levels was primarily affecting the export and not the splicing of the hRLUC reporter. This could be because the remaining 50% of the protein is sufficient to carry out its functions in spliceosome assembly but falls short in recruiting export factors. This preferential effect of UAP56 depletion on SPLCX demonstrates its responsiveness to splicing-related export deficiencies. The stable cell lines carrying a single copy of the SPLCX construct failed to show a similar response to UAP56 depletion (data not shown). Residual levels of UAP56 could be enough for the processing of the single copy transgene in the stable cell lines. Transient transfections expose the cell to multiple copies of the constructs and depleted UAP56 levels may be insufficient to cope with the increased demand for pre-mRNA processing.

In order to confirm the sensitivity of SPLCX, we depleted two additional co-transcriptional processing factors – nuclear export factor 1 (NXF1/TAP) and alternative splicing factor/splicing factor 2 (ASF/SF2). Northern blot analysis of mRNA levels demonstrated a 22% and 24% decrease in NXF1 and ASF respectively (Fig. 5A). The modest level of knockdown obtained for these proteins may reflect their importance for cellular survival. Cells with a more severe knockdown of these vital proteins may not survive, resulting in a selected population of cells with protein levels adequate for survival. Transient transfection of BI-16 and SPLCX into NXF1 depleted cells showed a significant decrease ($p<0.05$) in hRLUC/FLUC ratios for SPLCX not BI-16, as compared to wildtype cells. On the other hand, knockdown of ASF to similar levels did not yield similar results. Neither SPLCX nor BI-16 showing a significant change in hRLUC/FLUC ratios. This absence of an effect could be because ASF is a splicing factor belonging to the SR protein family. SR proteins are known to be functionally redundant, and other splicing factors like SC35 have been previously shown to successfully replace ASF in splicing reactions [33;34]. It is also possible that the modest decrease in protein levels may be insufficient to see an effect, as the residual ASF is capable of fulfilling its physiological function.

Conclusion

Splicing is emerging as a pivotal co-transcriptional process that links the nascent mRNA to the export machinery and the eventual transition from transcription to translation in metazoans [10]. As we learn more about the intricate network of proteins involved in the complexes linking the splicing and export of mRNA, there arises a need for sensitive, high throughput assays to

evaluate the roles of various proteins involved in these processes. Splicing reporter systems have been used in the past to study aberrant splicing [35;36], but those studies focused primarily on alternative splicing and exon skipping as a result of cis-acting mutations in single genes. Here, we have designed a reporter system that can be used to study defects in RNA metabolism caused by deficiencies in proteins involved in splicing-mediated co-transcriptional events. We have demonstrated the sensitivity of the assay by transfecting the reporter construct into cells deficient in three different co-transcriptional proteins. Depletion of UAP56 to 50% of wildtype levels resulted in the decreased nuclear export and expression of the splicing reporter. A modest decrease was sufficient to see a phenotype from NXF1 depleted cells, but did not elicit a response from cells with similarly decreased levels of ASF. The response seen in the NXF1 knockdown cells indicates that the splicing reporter is sensitive enough to detect an effect in the window of protein depletion before cell viability is affected. Transfecting the construct into various cell lines would provide a quick screen of any splicing/export deficiencies resulting from depletion or lack of a target protein. The ratiometric output reduces variability due to differing transfection efficiencies. Furthermore, other splice acceptor and donor pairs of interest can be easily cloned into the construct in order to analyze a different subset of processing proteins. The BI-16 construct and FLUC expressed from SPLCX under control of the same promoter as hRLUC provide excellent controls in our system. The assay produces a self-controlled ratiometric luciferase readout, which provides a sensitive and reproducible, albeit indirect measure of splicing and related processes via a simple reporter assay. Our system provides an easy measure of effective gene expression and is suited for high throughput analysis.

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Fig. 1. The SPLCX construct splices effectively

A. Schematics of Bi-16 (intronless control construct) and SPLCX (intron-bearing construct for splicing and export). Both constructs express firefly (FLUC) and renilla (hRLUC) reporters, driven by a bidirectional tetracycline inducible promoter. SPLCX contains the splice donor and splice acceptor regions from the *FXN* first intron upstream of the hRLUC reporter.

B. RNA was prepared from HEK 293 cells that were transfected with the SPLCX construct. Gel image shows that PCR amplification of reverse transcribed (+RT) RNA using primers flanking the splice sites produced a robust band of the expected spliced size. This band was purified and sequenced. No unspliced or mis-spliced products were seen.

C. Sequence of the spliced PCR product showing the expected frataxin partial peptide and the location of the splice junction. The translation re-initiation leader features two stop codons in each frame (underlined) and a Kozak consensus sequence in bold at the ATG.

Fig. 2. Splicing enhances nuclear export of hRLUC mRNA

Real time PCR analysis of FLUC and hRLUC mRNA levels in the nuclear and cytoplasmic compartments of BI-16 and SPLCX cell lines. A significantly increased level of hRLUC mRNA was observed in the cytoplasm for SPLCX when compared to the nuclear fraction. BI-16 did not demonstrate a similar distribution of the intronless hRLUC mRNA. Error bars represent the SEM of 4 independent experiments and * indicates a $p < 0.05$.

Fig. 3. Splicing enhances hRLUC expression in the SPLCX construct

Luciferase expression from BI-16 and SPLCX were analyzed in transient transfections and stable cell lines. The average hRLUC/FLUC ratios for SPLCX transient transfections and stable cell lines is shown compared to the BI-16 control that has been normalized to 1. A significant increase of approximately 2.4 and 2.8 fold in average hRLUC/FLUC ratios was observed when comparing SPLCX to the Bi-16 control construct in transient transfections and stable cell lines respectively. Error bars indicate the standard error of mean (SEM) of 12 replicates from 4 independent experiments and * indicates a [$p < 0.05$].

Fig. 4. Depletion of UAP56 decreases nuclear export of hRLUC from the splicing construct

A. Depletion of UAP56 by shRNA. Anti-UAP56 antibody (Abnova) produces a doublet, shown here. Western blots showed a ~50% decrease in the higher band in cells transfected with a vector expressing a UAP56-targeting shRNA, when compared to cells transfected with the empty pLKO.1 vector. The lower band also shows a marked decrease in intensity. Beta-actin was used as a loading control. Knockdowns were done 4 times with a representative blot shown. B. UAP56 depletion decreases hRLUC/FLUC ratio from SPLCX. Dual luciferase assays from transient transfections carried out after knocking down UAP56 (+shRNA) demonstrate a significant decrease in hRLUC/FLUC ratios in SPLCX when compared to cells with wildtype

levels of UAP56 (+pLKO.1). Error bars represent the SEM of 18 replicates from 6 independent experiments and * indicates a $p < 0.05$.

C. UAP56 depletion impedes nuclear export of the spliced SPLCX hRLUC mRNA. Nuclear and cytoplasmic fractions from UAP56 depleted cells transiently transfected with BI-16 or SPLCX were analyzed for FLUC and hRLUC mRNA levels using real time RT-PCR. No preferential nuclear export of hRLUC mRNA expressed from BI-16 or SPLCX is seen when UAP56 is depleted. Error bars represent the SEM of 3 independent experiments.

D. Northern blot analysis of total mRNA levels of hRLUC and FLUC transcribed from BI-16 and SPLCX in wildtype and UAP56 depleted cells. Wildtype and UAP56 depleted cells electroporated with either BI-16 or SPLCX were analyzed for unspliced mRNA products. Some unspliced message was observed in both SPLCX transfected cell types.

E. Percent unspliced SPLCX hRLUC mRNA in wildtype and UAP56 depleted cells. Densitometric analysis showed no significant increase of the unspliced product in UAP56 depleted cells when compared to wildtype cells.

Fig. 5. Depletion of NXF1 and ASF have different effects on the splicing construct

A. shRNA-mediated depletion of NXF1 and ASF. Northern blot analysis was carried out on wildtype cells and cells transfected with NXF1 or ASF shRNA. Densitometric analysis showed a 22% and 24% decrease of NXF1 and ASF respectively, when compared to wildtype cells. GAPDH mRNA was used for normalization. The bars represent residual mRNA levels expressed as a percentage of wildtype levels.

B. NXF1 depletion decreases hRLUC/FLUC ratios of the splicing construct. Dual luciferase assays from transient transfections carried out after knocking down NXF1 (+shRNA) demonstrate a significant decrease in hRLUC/FLUC ratios in SPLCX when compared to cells with wildtype levels of NXF1 (+pLKO.1). Depletion of ASF does not decrease hRLUC/FLUC ratios. Error bars represent the SEM of 3 independent experiments and * indicates a $p < 0.05$.