A Pan-Cancer Analysis of Transcriptome Changes Associated with Somatic Mutations in U2AF1 Reveals Commonly Altered Splicing Events

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Abstract

Although recurrent somatic mutations in the splicing factor U2AF1 (also known as U2AF35) have been identified in multiple cancer types, the effects of these mutations on the cancer transcriptome have yet to be fully elucidated. Here, we identified splicing alterations associated with U2AF1 mutations across distinct cancers using DNA and RNA sequencing data from The Cancer Genome Atlas (TCGA). Using RNA-Seq data from 182 lung adenocarcinomas and 167 acute myeloid leukemias (AML), in which U2AF1 is somatically mutated in 3–4% of cases, we identified 131 and 369 splicing alterations, respectively, that were significantly associated with U2AF1 mutation. Of these, 30 splicing alterations were statistically significant in both lung adenocarcinoma and AML, including three genes in the Cancer Gene Census, CTNNB1, CHCHD7, and PICALM. Cell line experiments expressing U2AF1 S34F in HeLa cells and in 293T cells provide further support that these altered splicing events are caused by U2AF1 mutation. Consistent with the function of U2AF1 in 3’ splice site recognition, we found that S34F/Y mutations cause preferences for CAG over UAG 3’ splice site sequences. This report demonstrates consistent effects of U2AF1 mutation on splicing in distinct cancer cell types.

Introduction

Recent whole-exome sequencing studies have identified recurrent somatic mutations in splicing factors in myelodysplastic syndromes [1], chronic lymphocytic leukemia [2], acute myeloid leukemia [3], breast cancer [4], lung adenocarcinoma [5], and uveal melanoma [6]. Many of these mutated splicing factors are branch point or 3’ splice site recognition factors (e.g., SF3B1 and U2AF1) and are involved in regulating intron removal from pre-mRNA. The 3’ splice site recognition complex is composed of U2AF1 and U2AF2 (also known as U2AF55), where U2AF1 is important for the recognition of the AG at 3’ splice sites [7] and U2AF2 recognizes the polypyrimidine tract upstream of the AG [8].

U2AF1 has been reported to have significant hotspot mutations at amino acid position 34 in myelodysplastic syndromes [1], lung adenocarcinomas [3], and AML [3]. U2AF1 mutations co-occur with mutations in known driver oncogenes in lung adenocarcinoma [5] and it is yet unclear if these mutations are oncogenically transforming. To further elucidate core effects of U2AF1 mutations on cancer biology, we aimed to identify common transcriptome alterations associated with U2AF1 mutations in distinct cancer types.

Results

Somatic mutations in U2AF1 across 12 cancer types

To look at transcriptome changes associated with U2AF1 mutations in different cancer types, we used The Cancer Genome Atlas (TCGA) data that provide both somatic mutations at the DNA level and high-throughput sequencing of mRNA (RNA-Seq) in the same individual specimens across multiple cancer types. Using available data from 12 TCGA cancer types [9], we identified cancer harboring somatic mutations in U2AF1. Across 2,979 cancer specimens with both whole-exome sequencing and RNA-Seq, 26 had missense mutations in U2AF1—17 at amino acid position 34 (Table S1 in Document S1, Figure 1). U2AF1 S34F mutations were found in eight lung adenocarcinomas (4%), four AML samples (2%), two endometrial carcinomas (1%), and one bladder cancer sample (1%). There were also two U2AF1 S34Y AML samples (1%). U2AF1 was reported to be significantly mutated in both lung adenocarcinomas [5] and AML [3], however, not significantly mutated in endometrial carcinomas.

Changes in splicing in U2AF1 mutations

Using available data from 12 TCGA cancer types [9], we identified cancer harboring somatic mutations in U2AF1. Across 2,979 cancer specimens with both whole-exome sequencing and RNA-Seq, 26 had missense mutations in U2AF1—17 at amino acid position 34 (Table S1 in Document S1, Figure 1). U2AF1 S34F mutations were found in eight lung adenocarcinomas (4%), four AML samples (2%), two endometrial carcinomas (1%), and one bladder cancer sample (1%). There were also two U2AF1 S34Y AML samples (1%). U2AF1 was reported to be significantly mutated in both lung adenocarcinomas [5] and AML [3], however, not significantly mutated in endometrial carcinomas.
most likely due to the low frequency within this cancer type. In addition to S34F/Y mutations, eight other somatic mutations were observed in U2AF1. To identify transcriptome alterations associated with U2AF1 mutation, we focused on lung adenocarcinoma and AML as these cancer types have a higher frequency of mutations and thus would have more power to detect statistically significant changes associated with the mutation and control for differences between tissue of origin. The S34F and S34Y mutations result in aromatic amino acids; therefore, we consider S34F and S34Y mutations to be functionally similar.

Transcriptome alterations associated with U2AF1 S34F/Y mutations in lung adenocarcinoma and acute myeloid leukemia

We used JuncBASE [11] to identify alternative splicing events that were significantly differentially spliced between U2AF1 wild-type cancer samples and U2AF1 S34F/Y samples. JuncBASE identifies and classifies forms of alternative splicing (e.g., cassette exon, alternative 5' splice sites, alternative 3' splice sites), quantifies the abundance of the inclusion or exclusion of each alternative exon, then calculates a “percent spliced in” (PSI) value for each splicing event in each cancer sample. A Wilcoxon rank-sum test was performed on PSI values between cancers with or without U2AF1 S34F/Y mutations to identify splicing events that were significantly different between the two groups. To control for tissue of origin, we compared U2AF1 wild-type and S34F/Y samples within the same cancer type. The U2AF1 S34F lung adenocarcinomas were found across the three expression subtypes (TCGA, submitted) and the U2AF1 S34F/Y AML specimens occurred across five French-America-British (FAB) AML subtypes [3]; therefore, we did not further control for subtype. Somatic mutations in U2AF1 have been found to be mutually exclusive with other splicing factors [1,3,5], suggesting that spliceosome pathway mutations may have the same functional effect on oncogenesis. To remove potential confounders from mutations in other splicing factors, we looked for differentially spliced events between samples with U2AF1 S34F/Y mutation and samples harboring no mutation in any splicing factor gene that has been

Figure 1. Splicing changes associated with U2AF1 S34F/Y mutation in lung adenocarcinoma and AML. (A) Representation of somatic mutations in U2AF1 observed across 12 TCGA cancer types [9]. Five cancer types had no somatic mutations in U2AF1. Amino acid positions are indicated. Zn, zinc finger; UHM, U2AF homology motif. (B) JuncBASE analysis of RNA-Seq data identified 131 and 369 splicing events significantly differentially spliced in lung adenocarcinoma and AML specimens bearing U2AF1 S34F/Y mutations, respectively.

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previously reported to be significantly altered in cancer (Tables S1–S2 in Document S1).

Using JuncBASE, we identified 131 and 369 alternative splicing events that were significantly differentially spliced in the presence of a U2AF1 S34F/Y mutation in lung adenocarcinoma and AML (False Discovery Rate (FDR)<5%), respectively. Additionally, these splicing events have a >10% difference in the median PSI of cancers with no splicing factor gene mutation and cancers with U2AF1 S34F/Y mutations (File S1–S3). Comparing U2AF1 S34F/Y mutation with U2AF1 wild-type samples (some with mutations in other splicing factors) yielded similar results in lung adenocarcinoma (TCGA, submitted) and AML (File S4). JuncBASE quantification of U2AF1 S34F/Y-associated splicing events in AML were consistent with an analysis of U2AF1 mutations in 20 AML samples [12](χ² = 0.84, Figure S1 in Document S1).

As both a positive and negative control for this analysis, we compared 10 lung adenocarcinoma samples with a MET exon 14 splice site mutation or deletion with all other lung adenocarcinoma samples to look for significant differential splicing between the two groups. The MET exon 14 alterations occur at a similar frequency as U2AF1 mutations in lung adenocarcinoma (TCGA, submitted). This analysis serves as a positive control, because the most strongly associated differential splicing event should be MET exon 14. This analysis also serves as a negative control as the alterations would not be expected to cause global changes in splicing since the mutations occur at cis-acting splice site alterations. Indeed, JuncBASE analysis only identified the splicing of MET exon 14 as significantly differentially spliced with a difference in PSI > 10%.

As an additional control, we identified alternative splicing events significantly associated with STAG2 mutations in lung adenocarcinoma and AML, as STAG2 is not expected to be a splicing regulator and it is mutated at a similar frequency in both cancer types. Only one splicing event was significantly associated with STAG2 mutation in lung adenocarcinoma and none in AML.

Similar to what was observed in a comprehensive characterization of lung adenocarcinoma (TCGA, submitted), the splicing events associated with U2AF1 S34F mutations in AML also show enrichment of cassette exons and alternative 3′ splice sites (Figure 1B). We do not observe broad effects on intron retention, which is thought to occur in the presence of a spliceosome mutation. If U2AF1 mutations cause many introns to be unspliced, perhaps it is not in a consistent manner and those instances are cleared away by nonsense-mediated decay (NMD). To see if the cassette exon and 3′ splice site bias is a general feature of alternative splicing in these cancer types or specific to splicing altered by U2AF1 mutation, we identified the proportions of each type of alternative splicing event in the top 10% most highly variable splicing events in lung adenocarcinomas without splicing factor mutations (Figure S2 in Document S1). U2AF1 S34F/Y cancers preferentially exhibit alterations in cassette exon and 3′ splice sites compared to the set of highly variable splicing events (chi-squared test, P = 6.6e-26).

Although there are splicing changes that are associated with U2AF1 S34F/Y mutation in these cancer specimens, it is unclear if the splicing changes are caused by the mutation or if there are unknown genomic alterations that are also correlating with the splicing changes. To evaluate further the relationship between the splicing changes and U2AF1 S34F mutations, we analyzed previously published RNA-Seq data comparing ectopic expression of U2AF1 S34F or U2AF1 wild-type expression in HeLa cells [1]. JuncBASE analysis comparing no-induction controls with U2AF1 wild-type or U2AF1 S34F induction identified 1,221 and 5,399 significant splicing changes, respectively (File S3 and S6). We find a significant overlap of 165 splicing changes upon induction of U2AF1 S34F in HeLa cells and splicing changes in human cancers with U2AF1 S34F/Y mutations (Figure 2, Fisher’s exact test, P< 2.2e-16). In contrast, we find a smaller proportion of splicing changes upon U2AF1 wild-type induction to be similar to alterations in human cancers (15/1221, Fisher’s exact test, P = 0.72). In total, 35% of the U2AF1 S34F/Y-associated splicing changes seen in lung adenocarcinoma or AML are supported by splicing changes caused by U2AF1 S34F expression in HeLa cells. We would not expect all splicing changes to be observed in the HeLa cell experiments as splicing changes are known to be context dependent [reviewed in [13]] and U2AF1 induction causes overexpression of the gene [1], which may affect spliceosome complex formation.

U2AF1 S34F/Y preferentially splices to CAG rather than UAG 3′ splice site sequences

U2AF1 is the small subunit of the 2AF heterodimer that recognizes the AG consensus of 3′ splice sites. A previous study that identified differential splicing of 35 exons associated with U2AF1 mutation identified a sequence signature at 3′ splice sites of the alternative exons [12]. Given the significant changes in cassette exons and alternative 3′ splice site events associated with U2AF1 S34F/Y mutation, we examined the sequences of 3′ splice sites that were preferentially used in U2AF1 S34F/Y mutated cancers. For simplicity, we only examined splicing changes between two alternate 3′ splice sites.

As splicing factor binding can differ depending on splicing activation or repression [reviewed in [14]], we looked at splice site sequences of exon inclusion and exclusion events, separately. From the set of U2AF1 S34F/Y-associated cassette exons and alternative 3′ splice site changes in AML, we found a significant bias toward skipping of an exon (69% of cassette exons) or usage of a more distal 3′ splice site (75% of alternative 3′ splice sites) (binomial test, P<1e-4, Figure 3A). We found a similar bias toward exon skipping and distal splice site usage of U2AF1 S34F-associated splicing events in lung adenocarcinoma (Figure S3 in Document S1). As a comparison, we identified alternative splicing events significantly associated with RBM10 mutation in lung adenocarcinoma (File S7). The RNA binding protein RBM10 was found to be significantly mutated in lung adenocarcinomas with recurring frameshift, nonsense, or splice site mutations [5]. The observed exon skipping is specific to U2AF1 S34F-associated splicing events.
as the opposite bias is observed in \textit{RBM10} loss-of-function (LOF)-
associated splicing events (Figure S4A–B in Document S1).

Consistent with previous findings \cite{12}, we found a trinucleotide
consensus TAG sequence at proximal 3’ splice sites of skipped
cassette exons. Moreover, we also find this motif at proximal splice
sites of alternative 3’ splice site events in \textit{U2AF1} S34F/Y AML
samples (Figure 3B). Additionally, a consensus CAG splice site at
the more distal 3’ splice site is observed, which has not been
previously reported (Figure 3B). A similar preference of CAG
splice sites (with a minor AAG sequence) over TAG splice sites is
observed in exons preferentially included in \textit{U2AF1} S34F/Y AML
samples (Figure 3C). These preferential splice site sequence motifs
differ from splice site motifs observed near proximal and distal 3’
splice sites of control alternative splicing events (Figure S4C in
Document S1, Wilcoxon rank-sum test, FDR \textgreater{}95%).

Strikingly, we see the same sequence preferences in cassette
exon and alternative 3’ splice site changes in \textit{U2AF1} S34F lung
adenocarcinomas (Figure S3 in Document S1) and in HeLa cells
transduced with \textit{U2AF1} S34F mutant constructs (Figure S5A–B in
Document S1). Moreover, we do not see this consensus 3’ splice
site sequence in \textit{RBM10} LOF-associated splicing in lung adeno-
carcinoma (Figure S4A–B in Document S1) nor in splicing
changes in HeLa cells transduced with wild-type \textit{U2AF1} (Figure
S5C–D in Document S1), supporting that the sequence preference
is specific to \textit{U2AF1} S34F/Y mutations.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Sequence preferences at altered 3’ splice sites associated with \textit{U2AF1} S34F/Y mutation. (A) Proportion of altered cassette exon and alternative 3’ splice site events that show exon skipping versus exon inclusion. (B) Consensus sequence motifs identified at the proximal (prox 3’ss) and distal 3’ splice sites (dist 3’ss) of exon skipping events. (C) Consensus sequence motifs at the proximal and distal 3’ splice sites of exon inclusion events. Splicing changes of expressed and annotated 3’ splice site choices where the splice site choice is TAG vs. CAG or TAG vs. AAG in (D) HeLa cells+induced \textit{U2AF1} S34F or (E) HeLa cells+induced \textit{U2AF1} wild-type. doi:10.1371/journal.pone.0087361.g003}
\end{figure}
These consensus sequence motifs were generated from splicing events that were strongly associated with U2AF1 mutation and do not resolve sequence preferences between two splice site choices from an individual splicing event. To further investigate the CAG splice site preferences over UAG splice sites, we examined splice site sequence switches between all pairs of expressed and annotated CAG versus TAG 3’ splice sites. Using a lowered threshold for identifying splicing switches, we found that 57% of CAG versus TAG 3’ splice sites were altered in HeLa cells expressing the U2AF1 S34F mutation, while 36% were altered when expressing a U2AF1 wild-type construct (Figure 3D). Of U2AF1 S34F-associated splice site switches, 83% (384/1,065) were a TAG to a CAG splice site, while only 53% (347/660) switched from a TAG to a CAG when expressing U2AF1 wild-type (Fisher’s exact test, P<2.2e-16). A similar bias of switches from TAG to AAG 3’ splice sites was observed (Fisher’s exact test, P = 2.518e-5, Figure 3D).

Our analysis shows that the U2AF1 S34F/Y mutation causes alterations in 3’ splice site usage where a C/AAG 3’ splice site is preferred over a UAG splice site. We did not observe altered splicing of all UAG 3’ splice sites; however, it is difficult to distinguish between unaltered splicing or degradation of the aberrant transcript by nonsense-mediated decay, thus appearing to be unchanged.

Transcriptome alterations in cell cycle genes and other cancer genes are associated with U2AF1 mutation

Somatic mutation in U2AF1 may cause splicing changes of key cancer genes, resulting in altered gene function or altered pathways. To identify potential downstream biological consequences of U2AF1 mutation, we performed a Gene Set Enrichment Analysis (GSEA) [15,16] to identify gene sets positively correlated with the U2AF1 S34F/Y mutation in lung adenocarcinoma and in AML. No gene sets reached statistical significance given an FDR cutoff of 25%. It was previously reported that transduction of U2AF1 S34F mutations in HeLa cells caused upregulation of components of the nonsense-mediated decay (NMD) pathway [1]. We did not observe a significant upregulation in expression of NMD factors associated with U2AF1 mutation in lung adenocarcinoma or AML.

Although no gene sets passed FDR significance, the top-two enriched GO ontology gene sets in AML with the highest normalized enrichment scores were “mitotic cell cycle” and “M phase of mitotic cell cycle” (Figure S6 in Document S1, nominal P<0.05). A subset of these cell cycle genes were upregulated in AML samples with U2AF1 S34F/Y mutation. Consistent with the effect of U2AF1 mutation on the cell cycle, RNAi depletion of U2AF1 [17] and U2AF1 S34F expression in HeLa cells [1] results in an increased fraction of cells in G2/M phase. We do not see any association with cell cycle gene sets in U2AF1 S34F lung adenocarcinomas. This may be explained by the high normal tissue contamination in this cancer type [18], which may affect gene expression signatures.

In line with these observations, there are 31 genes with U2AF1 S34F/Y-associated splicing events represented in the “mitotic cell cycle” and “M phase of mitotic cell cycle” gene sets, including CHEK2, CDC27, and multiple subunits of the anaphase-promoting complex (Table S3 in Document S1).

Splicing alterations associated with U2AF1 S34F/Y mutation in both lung adenocarcinoma and AML are of particular interest as these commonly altered genes may make core contributions to selective advantage in these cancers. We found a significant overlap of 30 altered splicing events that were associated with U2AF1 S34F/Y mutations in both lung adenocarcinoma and AML (Figure 2 and Table 1, Fisher’s exact test p<2.2e-16). For all 30 splicing events, the direction of splicing was the same in both cancer types, where U2AF1 mutation caused a shift to exon skipping or exon inclusion for the same splicing event. The majority (63%) of the commonly altered events showed more distal 3’ splice site usage in U2AF1 mutated samples. In addition, 17/30 splicing events were significantly affected upon induction of U2AF1 S34F in HeLa cells but had no change upon induction of U2AF1 wild-type (Table 1). 60% of these commonly altered splicing events show 3’ splice site sequence preference of a CAG, or AAG, splice site over a TAG splice site (Table 1, a or b), consistent with the overall observed splice site sequence preference of U2AF1 S34F/Y-associated events. Additionally, we looked for genes with altered splicing events that were also in the Cancer Gene Census [19] (Table S4 in Document S1). Three Cancer Gene Census genes were among the 30 commonly altered splicing events and are of particular interest: CTNNB1 (TCGA, submitted), CHCHD7, and PICALM (Table 1 and Table S4 in Document S1).

The altered splicing event in CTNNB1 in U2AF1 mutant cancers results in a shift toward splicing of a more proximal splice site in the 3’ UTR of CTNNB1 (Figure 4A–B). Significant upregulation of proximal splice site usage was also observed in HeLa cells expressing U2AF1 S34F (Figure 4C). The upregulated isoform has been shown to be a less stable mRNA in HeLa cells [20]; therefore, the altered splicing event may result in a reduction of CTNNB1 protein levels. Although, CTNNB1 activation is a canonical driver mutation, a decrease in normal levels of CTNNB1 may also be advantageous to cancer cells as RNAi depletion of CTNNB1 has been shown to increase cell migration [21].

To further test experimentally whether the splicing events observed in U2AF1 mutant cancers are directly caused by U2AF1 mutation, we transfected 293T cells with either U2AF1 wild-type or U2AF1 S34F constructs and assayed changes in five splicing events using quantitative RT-PCR (Figure 5, Figure S7 in Document S1). We were able to validate changes in splicing in CTNNB1, CHCHD7, and PTBP1 in cells transfected with U2AF1 S34F constructs and not in cells transfected with U2AF1 wild-type (Figure 5, unpaired t-test, P<0.1). Splicing events in KARS and CHEK2 did not validate and may be context dependent, false-positives in the transcriptome analysis, false negatives in the transfection experiment, or indirect delayed targets of U2AF1 activity.

Discussion

Our analysis of splicing and gene expression changes associated with U2AF1 mutation across multiple cancer types has revealed alterations in 3’ splice site sequence recognition and highlights potential biological consequences through altered cancer genes, such as CTNNB1, PICALM, and CHCHD7. In addition, we have validated that at least some of these splicing alterations are direct consequences of U2AF1 mutation in transfection experiments.

Many factors are involved in correct splice site recognition in complex with U2AF1, including U2AF2. It has been well known that the 3’ splice site consensus motif includes a C or U nucleotide before the AG at the end of introns and mutations in U2AF1 give the first indication that a factor has a specific preference for this –5 position [12]. U2AF1 has been shown to weakly bind to RNA via it’s UHM domain [22]. Perhaps the S34F mutation in the upstream zinc finger domain affects 3’ splice site sequence recognition via direct interaction with the pre-mRNA or through protein-protein interactions with U2AF2, hnRNP A1 [23], or DEK [24]. Further biochemical characterization of the U2AF1
S34F protein in complex with other proteins may lead to a more general understanding of 3′ splice site sequence recognition.

In summary, we found multiple genes including several known cancer genes with altered splicing in the presence of the U2AF1 mutation both in human cancer and in U2AF1 mutant transfected cells. The impact of these splicing alterations on gene function is an important topic for future investigation. Our identification of splicing changes significantly and consistently associated with somatic mutations in the U2AF1 splicing factor highlights the need for future study into the function of alternative splice forms of genes in order to fully understand the genomic alterations associated with cancer.

**Methods**

**Somatic mutations**

Somatic mutation calls from the 12 TCGA Pan-Cancer cancer types were used (doi:10.7303/syn1710680.4), with the exception of lung adenocarcinoma. Lung adenocarcinoma somatic mutation calls were taken from the TCGA lung adenocarcinoma analysis working group (TCGA, submitted).

**RNA-Seq processing**

RNA-Seq alignment files for 230 lung adenocarcinomas and 173 acute myeloid leukemias (AML) were available through CGHub (https://cghub.ucsc.edu). AML alignments downloaded from CGHub used an older version of the human genome reference (hg18); therefore, the AML BAM files were converted to FASTQ using SamToFastq [25] and then realigned against hg19 using TopHat [26] with the following parameters: "-G [Gencode v7 transcripts [27]] –mate-inner-dist 300 –mate-std-dev 500". One AML sample failed RNA-Seq re-alignment and processing and was excluded: TCGA-AB-2977. RNA-Seq alignments of lung adenocarcinoma samples used MapSplice [28].

**Table 1. 30 significant splicing alterations associated with U2AF1 S34F/Y mutations in both lung adenocarcinoma and AML.**

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CE, cassette exon; A3S, alternative 3′ splice site; ALE, alternative last exon; *, U2AF1 wild-type induction caused differential splicing, but in the opposite direction as U2AF1 S34F induction; Splice site preference in U2AF1 S34F/Y samples: a, TAG→CAG; b, TAG→AAG; c, other.
doi:10.1371/journal.pone.0087361.t001

S34F protein in complex with other proteins may lead to a more general understanding of 3′ splice site sequence recognition.

In summary, we found multiple genes including several known cancer genes with altered splicing in the presence of the U2AF1 mutation both in human cancer and in U2AF1 mutant transfected cells. The impact of these splicing alterations on gene function is an important topic for future investigation. Our identification of splicing changes significantly and consistently associated with somatic mutations in the U2AF1 splicing factor highlights the need for future study into the function of alternative splice forms of genes in order to fully understand the genomic alterations associated with cancer.
numbers DRR001796-DRR001799. Reads were aligned using TopHat [26] using Gencode v7 [27] as a transcript guide.

Splicing analysis

Lung adenocarcinoma samples, AML samples, and samples from the HeLa cell line experiments (HeLa+U2AF1 wild-type non-induced, HeLa+U2AF1 S34F non-induced, HeLa+U2AF1 wild-type induced, HeLa+U2AF1 S34F induced) were run through JuncBASE v0.6 [11] as three separate analysis sets. For lung adenocarcinoma and AML samples, the JuncBASE parameters used to identify alternative splicing events and calculate “percent spliced in” (PSI) values were the following: -c 2 -j [introns from Gencode v7] –jcn_seq_len 88. For the HeLa cell experiments, the JuncBASE parameters used were the following: -c 2.5 -j [introns from Gencode v7] –jcn_seq_len 202. For lung adenocarcinoma, Cufflinks [29] de novo transcript annotations were available to incorporate potentially novel exons in the splicing analysis (TCGA, submitted). UCSC knownGenes hg19 transcript annotation was used to define annotated exons [30].

To identify U2AF1 S34F/Y-associated differential splicing events in lung adenocarcinoma and AML, compareSampleSets.py of the JuncBASE v0.6 package was used with the following parameters: --thresh 10 --mt_correction BH --which_test Wilcoxon --delta_thresh 5.0. Splicing events with a significant difference (FDR < 5%) in PSI values between samples with no splicing factor mutation and samples with U2AF1 S34F/Y mutation were further filtered for events with a difference in the median PSI greater than 10%.

To identify splicing events affected by expression of U2AF1 S34F in HeLa cells, pairwise_fishers_test_getASEvents_w_reference.py was used to compare inclusion and exclusion isoform read counts between HeLa+U2AF1 wild-type non-induced sequenced library and HeLa+U2AF1 S34F-induced sequenced library with the following parameters: --thresh 25 --min_dpsi_threshold 5.0 --method BH --sign_cutoff 0.05. To identify splicing events specific to U2AF1 S34F induction, the event could not also be significantly different between the two controls or when comparing the U2AF1 wild-type non-induced to U2AF1 wild-type induced. This last restriction was imposed to distinguish splicing changes due to the S34F mutation.
affected splicing event that has any change upon \textit{U2AF1} wild-type induction to be non-specific to the \textit{U2AF1} mutation; however, we make note in Table 1 of the \textit{CTNNB1} event that also showed a splicing change in \textit{U2AF1} wild-type induction but in the opposite direction.

In cases where there are more than two alternatives for a splicing event (e.g., three alternative 3' splice site choices), the most significant differentially expressed isoform is reported.

To identify \textit{U2AF1} S34F/Y-associated differential splicing in both lung adenocarcinoma and AML, we checked for overlap in the genomic coordinates of the splice event from each cancer type. We found 31 splicing events that occurred at the same genomic location of the genes; however, the event in \textit{ASPH} was not the same splicing event upon manual review and was not included in Table 1. To test for the significance of the 30 commonly altered splicing events in lung adenocarcinoma and AML, we used a Fisher's exact test where the 2x2 table contained the number of splicing events affected and unaffected by the \textit{U2AF1} S34F/Y mutation in the two cancer types. Only splicing events that were expressed in both cancer types were used in the 2x2 table.

The list of Cancer Gene Census [19] genes were downloaded on May 31, 2013 to compare against the list of \textit{U2AF1} S34F/Y-associated splicing events.

\textbf{3' splice site consensus sequences}

The splice site sequence analysis used \textit{U2AF1} S34F/Y-associated differentially spliced cassette exons and alternative 3' splice sites where only two alternate splice sites were observed from the RNA-Seq data. The direction of splicing (exon inclusion or skipping) was determined by taking the difference in the median PSI value between samples with no splicing factor mutation and samples with a \textit{U2AF1} S34F/Y mutation. For example, if the median PSI was lower in the \textit{U2AF1} mutated samples, the alternative exon was preferentially skipped in the presence of the mutation.

3' splice site sequences from the proximal and distal splice sites of each splicing event were extracted from genomic coordinates by taking 20 nt upstream and 3 nt downstream of the intron/exon boundary. Splice site sequences were used as input to WebLogo 3 [31,32] to generate consensus motif logos. Motif logos were only generated for cases with 10 or more exon inclusion or exclusion events.

\textbf{Sequence preference at 3' splice sites}

Cassette exon and alternative 3' splice site events involving only annotated introns from Gencode v7 [27] were used to further examine 3' splice site sequence preferences of \textit{U2AF1} S34F when expressed in HeLa cells. Splicing events that were detectable with at least 25 reads and had only two splice site choices were considered. To look more globally at splicing switches, we used a lower threshold of a change in PSI >5%, no statistical cutoff, and no consideration of inclusion or exclusion of an alternative region, as our criteria for calling a splicing switch. HeLa cells expressing \textit{U2AF1} S34F or \textit{U2AF1} wild-type were compared to their matched non-induced controls to determine if a splicing switch occurred.

\textbf{Gene Set Enrichment Analysis (GSEA)}

Gene expression quantification from RNA-Seq data was taken from the TCGA lung adenocarcinoma study (TCGA, \textit{submitted}) and from the TCGA Pan-Cancer Analysis Group for AML samples (doi:10.7303/syn1681084.1). Gene expression for both sets were given as RSEM values [33] and were pre-filtered before performing Gene Set Enrichment Analysis (GSEA) analysis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Quantitative RT-PCR validation of splicing events affected by expression of \textit{U2AF1} S34F in 293T cells. A fold difference between total gene expression and the inclusion isoform of each splicing event was normalized by the fold difference of a no-transfection control sample to yield a relative inclusion level for each sample. The genomic coordinates of tested splice events in (A) \textit{CTNNB1}, (B) \textit{CHCHD7}, and (C) \textit{PTBP1} are given in Table 1. doi:10.1371/journal.pone.0087361.g005}
\end{figure}
The gene expression values were filtered to remove unexpressed genes (RSEM values<0) and genes with low variance (fold change between the minimum and maximum value >1.5). The GO annotation database (c.5.bp.v3.1 symbols.gmt) and the set of nonsense-mediated decay (NMD) factors listed in Yoshiida et al. 2011 [1] were used as gene set inputs to GSEA. Significance was derived from 10,000 permutations of the phenotypes U2AF1 S34F/Y vs wild-type.

**Data visualization**

Plots comparing PSI values in samples with no splicing factor mutation to samples with U2AF1 S34F/Y samples were created with the ggplot2 [34]. RNA-Seq coverage plots were generated by converting BAM files to bigWig files using samtools [25], the wiggles script from TopHat [26], wigToBigWig [30], and visualized using the UCSC genome browser (http://genome.ucsc.edu) [30].

**Validation experiments in 293T cells**

293T cells were transfected with V5 tagged U2AF1 WT or U2AF1 S34F constructs in biological triplicate and expression was confirmed by western blot (Figure S7 in Document S1). Quantitative RT-PCR was performed using Taqman Universal PCR Master Mix (Life Technologies) on cDNA from non-transfected and transfected samples. Taqman primers and probes for five splicing events were selected for constitutive regions of each gene to detect changes in total isoform expression and also selected for a splice junction specific to the inclusion isoform (Table S5 in Document S1). Custom probe sets were designed with the Custom TaqMan Assay Design Tool (Life Technologies) used for quantitative RT-PCR of splicing events. PCR System under standard conditions. The fold difference when necessary. Assays were run on the ABI 7300 Real-Time using the Custom TaqMan Assay Design Tool (Life Technologies) (Table S5 in Document S1). Custom probe sets were designed for each gene to detect changes in total isoform expression and also confirmed by western blot (Figure S7 in Document S1).

**References**


**Author Contributions**

Conceived and designed the experiments: ANB PSC MM. Performed the experiments: ANB PSC. Analyzed the data: ANB. Contributed reagents/materials/analysis tools: LW TS MI GS CSP AS MR JC MSL DSD GG. Wrote the paper: ANB MM.


doi:10.1182/blood-2013-01-480970


