Proteomic Evidence for In-Frame and Out-of-Frame Alternatively Spliced Isoforms in Human and Mouse

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Abstract—In order to find evidence for translation of alternatively spliced transcripts, especially those that result in a change in reading frame, we collected exon-skipping cases previously found by RNA-Seq and applied a computational approach to screen millions of mass spectra. These spectra came from seven human and six mouse tissues, five of which are the same between the two organisms: liver, kidney, lung, heart, and brain. Overall, we detected 4 percent of all exon-skipping events found in RNA-seq data, regardless of their effect on reading frame. The fraction of alternative isoforms detected did not differ between out-of-frame and in-frame events. Moreover, the fraction of identified alternative exon-exon junctions and constitutive junctions were similar. Together, our results suggest that both in-frame and out-of-frame translation may be actively used to regulate protein activity or localization.

Index Terms—Mass spectrometry, dual-coding genes, exon-skipping, protein isoform

1 Introduction

The cellular splicing machinery excises introns and joins adjacent exons to make mRNAs. Although the correct ordering and inclusion of exons is critical to the creation of functional transcripts, it is subject to variation by so-called alternative splicing (AS). Alternative splicing results in the exclusion or inclusion of exons and introns into different transcript isoforms from the same gene [1]. There are many different types of alternative splicing, but in mammals the most abundant event is exon-skipping [2]. This type of AS is characterized by the complete presence/absence of an exon in mature transcripts.

Interestingly, half of human and mouse exon-skipping events cause a downstream shift in the codon reading frame because the excluded exon is not a multiple of three in length [3]. These AS events commonly lead to premature termination codons (PTCs) and these transcripts are often degraded by a mechanism known as nonsense mediated decay (NMD; [4], [5], [6]). It has been argued that these frame-disrupting AS events have a major role in the control of gene expression via NMD. This model, known as Regulated Unproductive Splicing and Translation (RUST), proposes a coupling between unproductive AS events (those that result in frame-shifts and premature stop codons in the mRNA) and mRNA degradation through the NMD pathway [4], [7], [8]. However, it is also possible that the function of translated proteins is being regulated by frame-disrupting AS, with alternative protein isoforms having alternative activity levels or alternative localization (e.g., [9]). The possibly functional genes encoding transcripts with multiple reading frames have been called dual-coding genes [10], [11]. These studies have shown evidence for translation of human dual-coding genes by searching translated cDNA sequences against protein sequence databases [10].

Evolutionary conservation has been used extensively to find functionally relevant cases of exon-skipping [12], with many species-specific AS events thought to result from cellular errors that have no function [13]. While skipped exons that are multiples of three in length are over-represented overall relative to random expectations [3], the existence of many evolutionarily conserved AS events that lead to frame disruption [3], [11], [14], [15], [16] suggests that many of them may be functionally relevant. Indeed, the presence of conserved dual-coding genes has also been used to argue for their biological functionality [10], [11].

Despite the widespread occurrence of AS in mammals (e.g., [15], [17]) the evidence for translation of these alternative transcripts is still sparse on a genomic scale. Clearly, this task is complicated by the bias introduced by NMD. Out-of-frame transcripts degraded by NMD will never be detected by sequencing and therefore will be absent in alternative splicing databases. Exploration of human mass spectrometry data has detected tens [18] to hundreds [19] of alternative proteins, while there are at least forty thousand AS events detected in the human transcriptome [20]. This tremendous gap between transcriptomic and proteomic evidence for alternative splicing raises questions about the importance of

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AS in the creation of new proteins, as well as about the power of mass spectrometry platforms to detect such events.

In this study, a large number of mass spectrometry (MS) datasets from several human and mouse tissues were screened to estimate the frequency of exon-exon junctions involved in AS events that maintain or disrupt the reference reading frame. We focused on evolutionarily conserved events recently described in the literature [15], assuming that these events are unlikely to be splicing errors. In order to contextualize our findings, we also estimate the detection frequency of exon-exon junctions involved in non-conserved AS events [17] and the junctions between constitutive exons.

2 Materials and Methods

2.1 Database of Exon-Skipping Events

2.1.1 Reference Protein Sequence Database

We performed MS/MS searches against the reference proteomes of human and mouse. The reference protein sequences were obtained from the files CCDS_protein.20121025.faa and CCDS_protein.20071128.faa available at the NCBI-CCDS database. These files contained 27,523 and 17,704 coding genes from the human and mouse genome, respectively. Fig. S1a, which can be found on the Computer Society Digital Library at http://doi.ieeecomputersociety.org/10.1109/TCBB.2015.2480068, gives an overview of the bioinformatic workflow for this section. For the analysis of constitutive junctions we obtained the coordinates of exon-exon junctions in the files CCDS.20121025.txt (human) and CCDS.20071128.txt (mouse) and the amino acid sequence encoded by each exon in the files CCDS_protein_exons.20121025.faa (human) CCDS_protein_exons.current.faa (mouse, assembly Mm37.1). By combining information from these files, we found the coordinates of amino acids at the exon boundaries, i.e., exon-exon junctions, of the reference protein sequences. By randomly choosing one exon-exon junction coordinate per gene, we created a database of 18,320 and 16,889 exon-exon junctions for human and mouse, respectively. These coordinates were then searched in the MS/MS search result files, obtained with the reference protein sequences, to find significant peptide-spectrum matches (PSMs) spanning each coordinate (Fig. S1b, available in the online supplemental material).

2.1.2 Alternative Protein Sequence Database

We obtained the coordinates for exon-skipping events that are conserved among mammals (rhesus macaque, mouse, rat, and cow) from supplementary Table S4 in [15]. We obtained the human-specific and mouse-specific exon-skipping events from supplementary Table S3 in [17]. For the exons collected in Merkin et al., 2012 [15], we used Biomart-Ensembl web browser to find the genomic coordinates for the human exons. Each set of exon coordinates were mapped to genomic annotations from the NCBI-CCDS database (file CCDS.20121025.txt). Supplementary Datasets 1 and 2, available online, describe all exon-skipping events analyzed herein. We obtained the exonic nucleotide sequence from the reference human (Hg19) and mouse (NCBI37) genomes via the UCSC genome browser. These data were used to reconstruct the alternative transcripts, i.e. create a coding sequence without the skipped exon. We then used the program transeq from the EMBOSS package (http://emboss.sourceforge.net) to translate all reconstructed alternative transcripts, keeping the reference reading-frame of each gene. For the protein sequences obtained from transcripts with frame-disrupting AS events, only amino acids upstream of the first termination codon were considered in the MS/MS searches (Fig. S1c, available in the online supplemental material).

2.2 Mass Spectrometry Data Analysis

In total, 5,990,793 spectra from seven distinct human tissues and 8,303,412 spectra from six distinct mouse tissues were downloaded from PRIDE [21] and PeptideAtlas [22] (Supplementary Dataset 3, available online). The tissues were chosen to match the tissues analyzed in the RNA-seq studies that define AS events [15], [17].

MS/MS database searches were carried out using Mascot v2.4 (available from Matrix Science under license). Each experimental dataset was analyzed separately against the protein sequence database with the following parameters: tryptic cleavage specificity, up to two missed cleavages; carbamidomethylation of cysteine as fixed modifications and oxidation of methionine as variable modifications were common for all searches. When available, the precursor mass and ion mass tolerance were obtained from the articles describing the experiments. Otherwise, we used the following instrument-specific parameters: for most searches the precursor mass tolerance was set to 2 Da in LCQ or 1.5 Da in LTQ instruments and the ion mass tolerance was set to 1 Da in LCQ or 0.8 Da in LTQ instruments. No peptides outside of the instrument's detection range were included in the analyses.

We obtained the false discovery rate (FDR) using the target/decoy strategy [23] as

$$\mathrm{FDR} = \frac{2 \cdot \mathrm{FP}}{\mathrm{TP} + \mathrm{FP}},$$

where TP is the number of peptide-spectrum matches in the target database and FP is the number of peptide-spectrum matches in the decoy database above a particular score threshold. Each mass spectrometry data set was searched against the target human or mouse proteome (consisted of 27,523 and 17,704 sequences respectively) combined with the same number of shuffled decoy sequences created by the Perl script decoy.pl (http://www.matrixscience.com). The FDR varies in these different human and mouse experiments depending upon the instruments, samples, size of the data, etc. After inspection of the FDR distributions for each human and mouse MS experiment, we observed that peptide-spectrum matches with ion scores $\geq$29 had FDRs ranging from 0.16 to 6.98 percent in various data sets with an average of 1.81 percent.

Each spectrum was searched against our alternative protein sequence database. In the case of frame-preserving exon-skipping events, to report the alternative splicing event the peptide was required to match the junction between the two exons flanking the alternative exon. Peptides that do not span exon-exon junction site cannot distinguish the reference and the alternative proteins in these cases and were therefore not considered. In the case of frame-disrupting exon-skipping events, peptide matches at the junction between the two exons flanking the alternative exon or at sequences downstream of the skipped exon were used as evidence of alternative

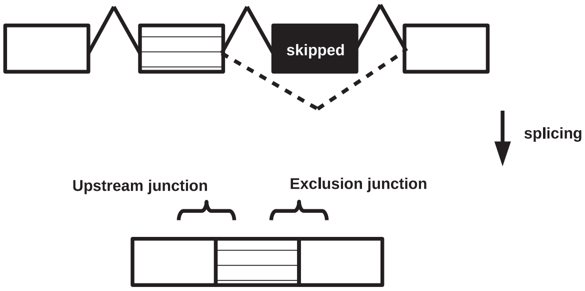

Fig. 1. Types of peptide spectrum matches under comparison. Boxes represent exons, connecting lines represent spliced out introns. The black box represents the skipped exon. Brackets represent PSMs. Upstream junction: Exon-exon junction upstream of the alternative exon. Exclusion junction: Junction reporting exon-skipping.

splicing. For these frame-disrupting cases, all amino acids downstream of the skipped exon are completely different from the reference sequence due to the shift in reading-frame. All significant spectra reporting alternative exon-exon junctions were manually inspected and annotated.

2.3 Gene Expression Data

Gene expression for 21,399 genes from eleven healthy human tissues was obtained from the RNA-Seq Atlas [24].

3 Results

3.1 Conserved Frame-Disrupting Events Drastically Shorten the Reference Protein Length

Evolutionary conservation is used as a proxy for several functionally relevant biological processes, and alternative splicing is not an exception. As previously described [3], [15], [17], [25], we found that at the transcriptional level there is a significant statistical association between the frame-preservation and the evolutionary conservation of the exon-skipping event ($P < 0.0001$, χ^2-test; Table S1a, available in the online supplemental material). The same pattern was observed for mouse; however, the significance was only marginal ($P = 0.05$; Table S1b, available in the online supplemental material). This depletion of frame-disrupting events among all conserved AS events likely reflects the action of natural selection removing splice variants that cause shifts in the reading-frame.

Nevertheless, at the transcript level, there are dozens of frame-disrupting events that are conserved among species diverged for more than 100 million years (see also [11]). Many of these conserved frame-disrupting events cause a drastic shortening of alternative isoforms relative to reference isoforms. We find that 20 percent of the conserved frame-disrupting events shorten the alternative protein to less than 100 amino acids. Conversely, frame-preserving events result in alternative proteins only slightly shorter than the reference in length (Fig. S2, available in the online supplemental material).

The evolutionary conservation of such frame-disrupting AS events suggests that they are unlikely to be products of splicing errors. However, these results do not tell us whether frame-disrupting events have a function at the transcriptomic or proteomic levels. Therefore, we searched major public repositories of mass spectrometry data for spectra reporting the exon-exon junction expected in case of exon skipping.

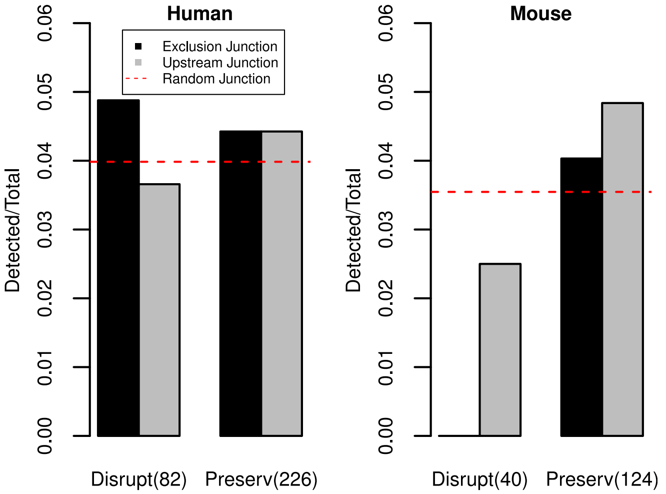

Fig. 2. Detection frequency of distinct exon-exon junctions as described in Fig. 1. a) Human. b) Mouse. Random Junctions: Exon-exon junctions randomly chosen from human or mouse reference proteomes (only one random junction per reference gene was drawn).

3.2 Estimating the Detection Frequency of Frame-Disrupting and Constitutive Events

We used the Mascot program to screen six million human and 8.3 million mouse spectra (Methods, Supplementary Dataset 3, available online). To detect exon-skipping events in MS data we searched for identified PSMs located at the junction between exons that flank the alternative exon (Exclusion junctions; Fig. 1). These PSMs allow us to find exon exclusion of frame-preserving or frame-disrupting exons. Importantly, all significant spectra reporting exclusion junctions were manually inspected. For comparison, we considered PSMs located in junctions of the same gene but immediately upstream (i.e., 5′) of the exon-skipping junctions (Upstream junctions; Fig. 1). The upstream junction set is made up almost entirely of constitutively spliced junctions.

In humans, we detected the exclusion of frame-disrupting exons in a total of four distinct genes from an initial database of 82 genes with detectable junctions (detection frequency: $4/82 = 4.9$ percent; Fig. 2a). Of these, two are evolutionary conserved. In mouse, no frame-disrupting events were detected among a total of 40 genes searched (detection frequency: 0 percent; Fig. 2b). The low detection frequency of frame-disrupting events could reflect a biological mechanism of degradation of transcripts with PTCs, or it could reveal a limitation of analytical platforms in detecting any kind of exon-exon junction. Favoring the latter hypothesis, the results show that, for both species, there is no significant difference in detection frequency between frame-disrupting and constitutive exon-exon junctions (3/82 and 1/40 upstream junctions were detected in human and mouse, respectively; $P = 1$, Fisher's exact test).

On a more technical note, it is important to further comment on the process of FDR estimation used here. It has been previously observed that the accuracy of estimated false discovery rates depends on several factors, including the number of identified spectra, mass tolerance, construction of the decoy database, and the choice of FDR estimators [26]. Consistent with these observations, over the 54 data sets searched in this study, we report that the same ion score threshold of 29 resulted in a relatively high negative correlation between the number of identified spectra and the FDR (Pearson's correlation of -0.47; $P = 4.0 \times 10^{-4}$). Moreover, while the choice of the FDR estimator is not always clear, the "original" formula $\mathrm{FDR} = \mathrm{FP}/\mathrm{TP}$ typically gives a less conservative estimate than the "alternative" formula $\mathrm{FDR} = 2 \cdot \mathrm{FP}/(\mathrm{TP} + \mathrm{FP})$ [26], [27]. Here we again observed similar trends; i.e. the ion score threshold of 29 resulted in $\mathrm{FDR} = 0.93\%$ using the original estimator, whereas the same score threshold resulted in $\mathrm{FDR} = 1.81\%$ using the alternative estimator. Overall, although the final identification results may be somewhat impacted by the decisions made in database search and FDR estimation, we believe that the spectral identification carried out in this work is of good quality, most likely with a true FDR around 1 percent.

3.3 Similar Detection Frequency among Frame-Disrupting, Frame-Preserving, and Random Exon-Exon Junctions

Given that the small size of the frame-disrupting database could lead to weak estimation of the detection frequency, we next focus on the detection frequency of a larger exon-exon junction database, consisting of frame-preserving and random junctions. The latter dataset consists of a set of junctions randomly selected from the reference proteome and therefore is enriched in constitutive junctions.

In humans, we detected the exclusion of frame-preserving exons in a total of 10 distinct genes from an initial database of 226 genes with detectable junctions (detection frequency: $10/226 = 4.4\%$; Fig. 2a). Again, no significant difference in detection frequency between frame-preserving and upstream junctions was observed. In mouse, the exclusion of frame-preserving exons was detected in 4.0 percent of genes (5/124; Fig. 2b). Interestingly, all 10 frame-preserving events detected in humans are evolutionarily conserved. Of the 226 genes containing frame-preserving events, 148 harbor evolutionarily conserved events and 78 do not. Therefore, for frame-preserving events, there is a significant association between MS detection and evolutionary conservation ($P = 0.01$, Fisher's exact test).

The detection frequency of random junctions was 4.0 percent (730/18320) in humans (Fig. 2a, dashed line) and 3.5 percent (599/16889) in mouse (Fig. 2b, dashed line). These frequencies are not significantly different from the detection frequencies of the exon-skipping events involving frame-preserving or frame-disrupting exons, for both species ($P > 0.5$ for all comparisons; χ^2-test). Therefore, we conclude that the estimation of the detection frequencies were consistent among larger and smaller exon-exon junction databases, and were similar regardless of the splicing type. Importantly, these conclusions hold even when only PSMs with $\mathrm{FDR} \leq 1\%$ are analyzed.

Supplementary Table S2, available online, summarizes features of the exon-skipping events we detected at the protein level; further information about each gene is also available in Supplementary Text 1, available online.

3.4 Low Frequency of Detected Exon-Exclusion Is Not Due to Low Gene Expression

The existence of a positive correlation between the gene expression and the level of protein detection on MS data have been described elsewhere [19], [28], [29]. By using Pearson's correlation we observe positive and significant correlations between gene expression (measured by RPKM) and protein detection (measured by the number of PSMs) ($P < 1.0 \times 10^{-7}$) for 6 tissue samples (Brain, Colon, Heart, Kidney, Liver, Lung) (Supplementary Dataset 4, available online). The best correlation was observed for the Liver sample (Fig. S3, available in the online supplemental material) where $R^2 = 0.19$, Pearson's correlation $= 0.44$, $P < 2.2 \times 10^{-16}$. Moreover, for these six tissues, the human genes containing the exon-skipping events (309 genes) showed a significantly higher level of expression than 100 sets of 309 genes randomly chosen from 20,048 human genes (Wilcox test, $P < 1.0 \times 10^{-24}$ for all comparisons). Additionally, the genes with conserved exon-skipping events are more highly expressed than the non-conserved ones. These results suggests that low gene expression cannot explain the low frequency of detected exon-skipping events in MS data.

4 Discussion

Since its discovery 40 years ago, alternative splicing has been seen as a biological mechanism for generating protein variability. Through RNA-Seq experiments [15], [17] hundreds of exon-skipping events shared among mammalian species have been discovered, with approximately 17 percent of them causing frame-disruption in both human and mouse genes. These events cause severe shortening of reference genes, producing highly truncated proteins.

Here we searched mass spectrometry data and found evidence for the translation of several of these out-of-frame isoforms (Table S2, available in the online supplemental material). Many of the exon exclusion events detected here at the protein level were previously detected at the transcript level by distinct methods, not only by RNA-seq (Supplementary text 2, available online). There is therefore good evidence that many of the events we have detected are real and biologically replicable.

The low detection frequency of truncated proteins found in mass spectrometry data must be considered against the low detection frequency of all splicing junctions, which was below 5 percent for both alternative and constitutive exons. The reasons for this low detection rate may be biological or methodological. Among the biological reasons, low protein abundance could account, at least in part, for this result. However, we found that most alternatively spliced genes analyzed here are highly expressed in several tissues, which likely indicates high protein abundance [29], [30] (Supplementary Dataset 4, Fig. S3, available online). Methodological limitations–such as the short length of the amino acid sequence required to detect an alternative exon-exon junction or low coverage of the proteome—could also explain the low detection frequency. Consistent with low protein coverage in bottom-up proteomics [31], our search of millions of spectra only resulted in the detection of about 30 percent of the human and mouse reference proteins when considering PSMs that mapped to any part of the protein sequence (Tables S3a and S3b, available in the online supplemental material). It should therefore not be surprising that when restricting the target matches to exon-exon junctions only, the detection frequency decreases to 4 percent.

Interestingly, this frequency is similar for alternative and constitutive exon-exon junctions. This surprising result suggests that translation of alternative transcripts may be more common than previously thought, at least in human and mouse. Importantly, the results presented here do not mean that frame-disrupting events are comparable in frequency to frame-preserving events, but rather that the rate at which junctions are detected in MS data seems to be proportional to their frequencies. This observation holds regardless of whether AS events cause frame disruption or whether the junction is constitutive or alternative.

Many authors have proposed that alternative splicing may be a mechanism for regulating transcript level via NMD by introducing PTCs into mature mRNAs [4], [6]. While there is good evidence supporting this mechanism [8], [32], our data suggest that some of these transcripts are translated (see also [33]). Regarding unproductive translation, we believe that the discovery of evolutionarily conserved truncated proteins suggests functional roles for these short proteins. Among the possible biological mechanisms regulated by truncated proteins are changes in cellular localization, for instance by the removal of nuclear signal peptides (e.g., [9]); or the creation of dominant negatives, for instance by proteins that can form heterodimers but are not enzymatically active (e.g., [34]). Any such proteins that can "quench" signals by receiving but not transmitting information are sure to be a useful tool in the regulation of cellular function.

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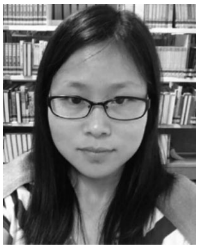

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