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Most Alternative Isoforms Are Not Functionally Important

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There are two assertions that might be considered polemical in our Opinion article [1]: the first is that most protein-coding genes have a single main isoform, and the second is that most annotated alternative transcripts do not generate functionally important gene products. While alternative isoforms can have important cellular roles, we believe that alternative splicing is not the key to proteome complexity.

In his letter [2] Professor Blencowe asks three rhetorical questions. The first is whether our results are surprising given the limitations of shotgun mass spectrometry (MS). Here Professor Blencowe can only be challenging the use of proteomics to support the existence of a single main protein isoform because our second assertion is not even partly based on the lack of proteomics evidence for alternative isoforms. The MS sensitivity argument is a red herring.

MS experiments do support the existence of a single main isoform for most genes. These main isoforms coincide almost perfectly with dominant isoforms predicted from conservation of protein structural and functional features and with variants agreed upon by manual genome annotators [3]. By way of contrast, where RNA level studies do find dominant transcripts [4], the overlap with the main proteomics isoform is remarkably low [3]. Part of the reason for this discrepancy is that RNA-seq transcript abundance algorithms are not good at estimating transcript abundance [5, 6].

Question two is ‘are the conclusions justified based on the findings?’ In our opinion the available evidence leaves little room for doubt. Most protein coding genes have main protein isoforms, and most alternative exons are subject to neutral or near-neutral evolution (Box 1). We believe our conclusions are well substantiated and invite readers to judge for themselves in the article and related papers [3, 7].

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Jose Manuel Rodriguez contributed to this letter.

The third question is the most interesting. Professor Blencowe asks what is the actual fraction of splice variants that are translated?' The short answer is that no-one knows. Clearly, as Professor Blencowe points out, shotgun MS evidence cannot be used; there are too many reasons for not detecting alternative variants. They may not be translated, they might be present in low abundance, or are only expressed in limited tissues, conditions, or developmental stages, or they might be translated and then quickly degraded.

Professor Blencowe claims that the detection of alternative transcripts at the ribosome together with correlation between protein and RNA levels demonstrates the wholesale translation of alternative transcripts. Unfortunately, this is not true either. First, at the transcript/isoform level protein–RNA correlation does not exist [6], and this argument therefore leads nowhere. Professor Blencowe's ribosome profile experiments [8], while interesting, do not guarantee translation either. The ribosome harbors an array of peptide quality-control mechanisms, as a recent review shows [9], and it is therefore not hard to imagine that a proportion of alternative variants may not survive scrutiny by the ribosome. Previous studies have shown that non-coding transcripts are also routinely detected in ribosome profiling experiments and there is a healthy scepticism about whether these code for stable proteins [10, 11].

Splice events identified in Professor Blencowe's ribosome profiling experiment differ from those found in large-scale proteomics experments. The exon skipping events interrogated in the ribosome profiling experiment were overwhelmingly novel (more than 90% are not annotated in evidence-based genome annotations) [8], and a large proportion would lead to frame changes, as Professor Blencowe points out [2]. By way of contrast just 3.75% of splice events detected in our proteomics experiments were frame-changing and many could be traced back to the earliest vertebrates [7], In fact the alternative isoforms we found almost always had intact functional domains [7], which strongly suggests some sort of selection either during or after translation.

Although genetic variation at the population level indicates that most alternative isoforms are unlikely to have meaningful functional roles (Box 1), this cannot tell us how many alternative transcripts are translated. Targeted proteomics (SRM) could provide some answers, but Professor Blencowe exaggerates its usefulness. Roest *et al.* [12] state that SRM 'can only monitor dozens to hundreds of peptides', while the targeted peptides in the study by Shreiner [13] identify only three pairs of splice isoforms, the long and short forms of neurexins *Nrxn1*, *Nrxn2*, and *Nrxn3*. These are conserved back to the earliest vertebrates, highlighting their importance, and we would therefore certainly expect these alternative isoforms to be translated. Indeed, both forms of *Nrxn2* are found in shotgun MS experiments of mouse brain [14].

The paper by Schreiner [13] does highlight the value of focusing efforts on exceptional biological cases. Researchers tend to make sweeping conclusions about genome-wide roles for alternative splicing when we actually know very little about the detailed functional roles of the vast majority of alternative isoforms, even those that are generated from highly conserved exons [7].

Finally, we believe that our Opinion article generates far more questions than answers. For example, why is there so much evidence for alternative transcripts, if most alternative exons are subject to neutral selection? Why do the alternative isoforms found in proteomics experiments maintain functional domain composition whereas most do not? What are the cellular roles of the alternative isoforms that are functionally important?

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Professor Blencowe cites a 2006 review in support of his contention that most alternative variants are functionally important, even though they are known to be under relaxed selection pressure. This is clearly an oversight. In 2006 researchers calculated selection pressures by aligning human DNA sequences with the genomes of related species such as mouse. Many alternative exons were found to be poorly conserved between species, but some researchers interpreted this lack of conservation as lineage-specific innovations.

A wealth of genome-wide genetic variation data from human populations has recently become available, enabling us to test whether alternative exons are undergoing purifying selection (whether they really are innovations). The variant data we analyzed were only available from 2012. These show that most alternative exons are evolving neutrally: they have a much higher non-synonymous to synonymous substitution ratio, and a 10-fold higher proportion of potentially damaging high-impact variants. Most interestingly, these figures do not decrease with increasing allele frequency, as would be expected if alternative exons were under selection pressure.

The neutral or near-neutral selection pressures apparent in the current population are a very strong suggestion that most alternative variants have not been evolutionary selected to have important cellular roles.