12/12/06
Dr. Greg Gibson
Associate Editor, *Genetics*

Dear Greg,

Thank you for the rapid and positive review of the manuscript "Mammalian mRNA splice-isoform selection is tightly controlled", #2006/066183. I am replying to the items mentioned in your review letter and in the individual reviewer comments. My apologies for the delay. Together with this letter, I am supplying a revised manuscript text and revised manuscript Figures. The revisions are described individually in this letter. I have also provided some supplemental information, as suggested by one reviewer. I do not believe that these supplemental data have sufficient value for interpreting the paper, but I am happy to add them to the document as an appendix, or have them accessible on the *Genetics* website, as you feel most appropriate.

Editorial comments:

1. "Reviewer #2 suggests adding some Q-RT-PCR data to potentially show that the alternative splice ratio stability occurs despite variation in abundance."

In addition to the large number of control reactions we have performed that define the sensitivity and reproducibility of our assay across a range of RNA concentrations, we have performed RT-PCR reactions on a set of animal RNA samples to estimate the relative abundance of the five primary RNA transcripts. The variation among the samples for transcript abundance is larger than the variation among the same samples for our splice-choice-ratio assay. We observe no significant correlation between the large inter-individual variation in the RT-PCR and the small inter-individual variation seen in splice-choice ratios. We have **modified the text** to reflect these data.

2. "A further description of the control methods, as requested by Reviewer #1."

We have now **added text** to the Results section describing our controls for the experiments. We feel that adding any Figures showing the controls or raw data would be of little added impact, but we can supply these if desired.

Reviewer #1 comments and our responses:

1. Examples from the literature of stringent alternative splicing control.

To our knowledge, no other study has been performed that directly answers the reviewer's question. The work of Baudry *et al.* (2000) and of Hagendorf *et al.* (2005) that we cite are the closest that we have found. However, the measurement strategies used in both papers are not as precise as our method. A less-precise assay method is likely to lead to observation of higher interindividual variation in RNA isoform ratios. We have also added a reference to a recent review paper by J.H. Marden (2006) in which the author states: "Few studies have examined how AS [alternative splicing] varies in a quantitative manner among individuals and populations, and to my knowledge, the heritability of quantitative variation in AS has not yet been determined for any gene in any organism."

2. Issues of global control of splicing; gene-to-gene differences in stability control; and differences in genes without alternative proteins.

The reviewer's questions are very similar to our own. We are currently performing a set of experiments to address several of the issues. However, essentially all of our existing data are presented in this paper. We do not know, as yet, if other transcription units will show the same

tight control as the five shown here. We are looking at another group of six alternatively spliced genes, and two of these six genes are not known to have protein-encoded differences between the alternative splice products.

3. Questions about methods, data for the controls, controls by mixing of cDNAs.

We have **modified the text** to address some of these concerns, and have supplied some supplemental results, as requested. Our assay system is highly reproducible and is precise. Replicate assays of the same initial RNA sample have very small variation, either when prepared at the same time, or across several months. We monitor reproducibility by including an aliquot from a single RNA sample in every assay tray, in triplicate. This single RNA sample has been measured for the splice choice ratio of each gene (reverse transcription plus PCR), in triplicate, at least six separate times. Our generalized statement of "1 part in 25" actually reflects the "worst case" of this testing. The **supplemental data** show the two most variable genes *Ezh2* and *Kras*, using a full tray (31 test animals) repeated twice. Only a single sample exceeds the 95% confidence intervals, in one gene, *Kras*. Each of the other genes has <u>less</u> replicate variation.

4. Statistical significance of the comparison between UM-HET3 vs. F1 hybrid animals.

We felt that given the low number of F1 animals that we measured (N = 9), the results should be considered primarily as a qualitative comparison. Therefore, we simply show the values for each of the individual animals in Figure 4. Using non-parametric tests (with Bonferroni correction for six analyses), neither the means nor variances are significantly different between the UM-HET3 and F1 hybrid groups.

5. Selection of the 30 late-life animals for age comparison.

The 30 animals for the subset 18-month-old individuals for age-dependent change were selected at random, without using any previously known information. This has been entered **into the text** in the Materials and Methods section.

6. Evidence linking protein abundance with mRNA splice variant abundance.

The reviewer is correct, that we can only imply a relationship between splice isoform ratios and the ratios of the final proteins. We do not have experimental data to confirm this assumption. We have **modified the text** as requested to reflect this.

7. Changes to Figures for clarity.

We have modified the Figures as suggested by the reviewer.

<u>Reviewer #2 comments and our responses:</u>

1. Quantitative analysis to determine the interindividual variation in transcript levels.

We have performed several control experiments that are mentioned in the text (see Editorial Comment 1).

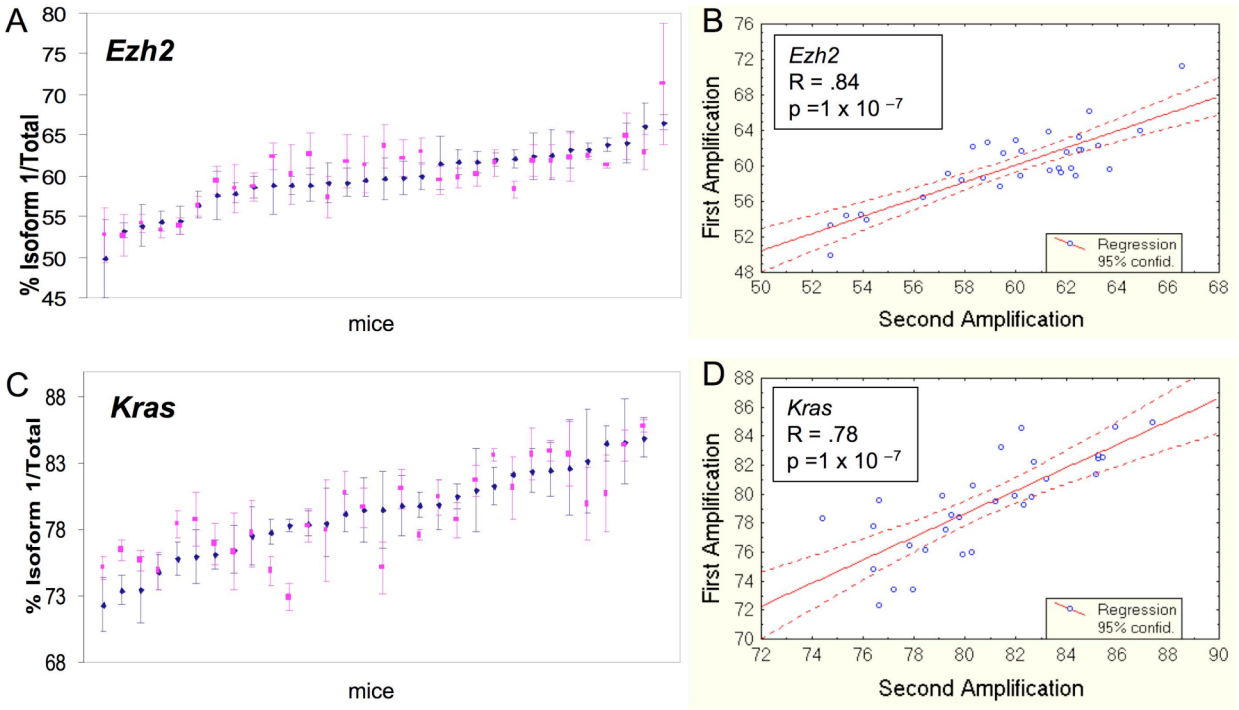
2. Figures and color.

As suggested, we have reduced the number figures that require the use of color.

3. Inclusion of literature reference for transcript abundance.

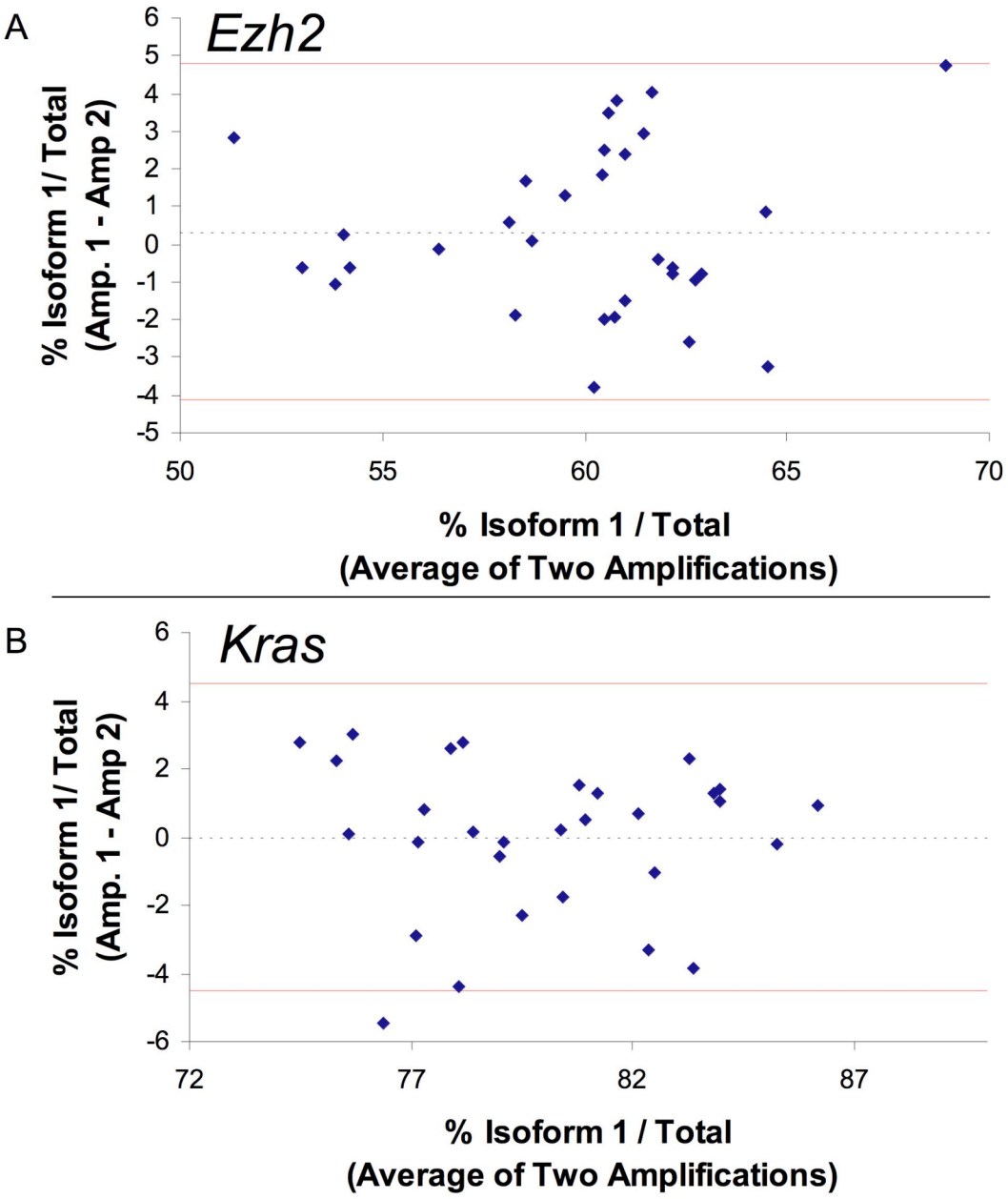
Although this is an interesting paper regarding RNA populations and aging, we felt that the reference did not substantially add to our discussion about alternative splice site choice.

Supplemental information: Chisa and Burke



Reproducibility of *Kras* and *Ezh2* Assays. *Kras* and *Ezh2* assays were repeated on one experimental tray (31 animals). Panels A and C show the mean of the PCR triplicates of the isoform ratio from both amplifications for each animal. Error bars show the S.E.M. for each value. The mice are ordered in increasing value of the first amplication, and the values from both the from the first amplification (◆) and the second amplification (■) are plotted. Panels B and D show the same information as A and C, in a scatterplot. The Pearson Product-Moment Coefficient is given, to show the degree of agreement between the repeated measures.

Supplemental information: Chisa and Burke



Repeatability of *Ezh2* and *Kras* Assays . *Kras* and *Ezh2* assays were repeated on one experimental tray (31 mice). The mean splice-isoform ratio from the two amplifications is plotted on the X-axis for each mouse. The Y-axis plots the ratio(amplification 1) – ratio(amplification 2) for each mouse. The dashed line is the mean difference in ratio between amplifications for all animals, and the red lines represent +/- 2 s.d. of the differences between amplifications (+/- 4.46 for *Ezh2* and +/- 4.48 for *Kras)*.