Dear Robert and Martin,

Thank you for your responses to my concerns and for posting these online. Also, thanks for clarifying that GRO/PRO-seq data were not used to define loci with bidirectional transcription initiation events. However, I still have some concerns over conflicting results that I believe mainly arise from different strategies on how to define bidirectional transcription and your lack of dealing with sequencing noise.

In order to not make this document too long for readability reasons, I decided to quote and respond to selected parts of your response to my original comments below.

"In your paper you looked for bidirectional transcription at enhancers and found it. Many of them (~70%) validated in enhancer assays. What you did not do, was to ask if that same signature of bidirectional transcription occurred at other, clearly non-enhancer sites and this is where we extend your observations".

This is not correct, we (Andersson et al. 2014) predicted enhancers from CAGE data alone and validated randomly selected loci. We did first check for the presence of enhancer RNAs at P300-centered chromatin-predicted enhancers and found a divergent pattern with balanced bidirectional transcription on both strands. We observed that this property was clearly different from the unbalanced bidirectional transcription pattern of RefSeq mRNA TSSs (biased to the sense strand). Based on this observation we devised a strategy to predict enhancers genome-wide from CAGE data alone. Our final set of predicted enhancers were loci with balanced bidirectional (divergent) transcription and were not selected based on overlap or proximity with ChIP-seq peaks or other data indicative of regulatory elements. Among predicted enhancers (from CAGE data alone) we randomly selected loci for validation in enhancer reporter assays and among those ~70% showed significant reporter activity compared to control. In contrast, only around 30% or less of predicted enhancers from chromatin data were positively validated (Kwasnieski et al., 2014; Kheradpour et al., 2013; Andersson et al., 2014).

"[The] original purpose of our study [was] to test whether the traditional enhancer definition using histone modifications was outperformed by transcriptomic approaches (Andersson et al. 2014). By intersecting chromatin state maps with evidence for bidirectional and unidirectional transcription, we showed that the chromatin state was a better predictor of enhancer activity both in reporter assays and by *in vivo* correlations of enhancer transcription with that of putative target promoters."

"We show that, on the much wider scale afforded by the Kwasnieski data set, chromatin states clearly separate DNA sequences that can behave more effectively as an enhancer while bidirectional transcription cannot".

From your wording above, it is easy to get the impression that you tested whether our approach to predict enhancers from bidirectional transcription (Andersson et al. 2014) outperforms chromatin-based approaches according to Kwasnieski et al's assay data. I therefore checked for overlap between tested regions of Kwasnieski et al (Kwasnieski et al., 2014) and our predicted K562 enhancers from the FANTOM5 project (Andersson et al., 2014) (894 enhancers with significant expression compared to random genomic regions in at least one out of three replicates of FANTOM5 K562 CAGE data). Surprisingly, none overlapped. There is thus a strong disagreement between your approach of identifying bidirectional transcribed loci and

the approach used by us in FANTOM5. I then quantified the number of CAGE tags from each FANTOM5 K562 CAGE library overlapping Kwasnieski et al regions (midpoints +/- 250bp of weak enhancer state regions (K_WS, K_WC), strong enhancer state regions (K_SS, K_SC), and repressed/low activity state regions (K_RC)). On average (between CAGE replicates), ~92% of weak enhancer state regions, 82-87% of strong enhancer state regions, and ~94% of repressed state regions had no overlapping CAGE tags. These results suggest that the regions tested by Kwasnieski et al, in general, do not represent transcription start sites in K562 cells.

So, how do the FANTOM5 enhancers compare with ENCODE chromatin state segmentations overall? I overlapped the FANTOM5 CAGE-predicted K562 enhancers with ENCODE K562 state segmentations (same set that was used by you in your study) and found 59% to overlap with predicted promoters, 27% with predicted enhancers, 4% with predicted transcribed regions, 3% with predicted repressed activity, and 6% to not overlap any state.

From these results I draw two conclusions. First, all chromatin states are not pervasively transcribed as you suggest in your preprint. Predictions of enhancers from bidirectional transcription (as defined in (Andersson et al., 2014)) do not generally coincide with regions of predicted repressed activity (3% do overlap but 84% of segmented genomic sequence falls into this predicted state) but are biased to predicted enhancer and promoter states (for a discussion about the similarities between enhancers and promoters see (Andersson et al., 2015)). Secondly, the vast majority of regions tested by Kwasnieski et al. have no evidence for transcription initiation events.

"While we could consider only bidirectionally transcribed enhancers with multiple reads on each strand this would only function to limit sample size and does not alter our conclusions."

I had a quick look at your Methods section again and can see that you did merge a large number (16) of CAGE libraries into one:

"CAGE data produced by the FANTOM5 consortium were downloaded in BAM format from http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeRikenCage/ and all libraries from each cell type were then merged into a single BAM file."

This means that a single CAGE tag from each strand in one locus might very likely come from different libraries and represent sequencing noise. (Also, note that the CAGE libraries you considered were not produced within FANTOM5 but by RIKEN for the ENCODE project).

Again, thank you for your detailed response. I hope that my comments might help explain some of the conflicting results between our studies

Robin Andersson, 2016-05-09

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