SEESAW: detecting isoform-level allelic imbalance accounting for inferential uncertainty

Euphy Y. Wu1, Noor P. Singh2, Kwangbom Choi3, Mohsen Zakeri2, Matthew Vincent3, Gary A. Churchill3, Cheryl L. Ackert-Bicknell4, Rob Patro2 and Michael I. Love1,5*

Abstract
Detecting allelic imbalance at the isoform level requires accounting for inferential uncertainty, caused by multi-mapping of RNA-seq reads. Our proposed method, SEESAW, uses Salmon and Swish to offer analysis at various levels of resolution, including gene, isoform, and aggregating isoforms to groups by transcription start site. The aggregation strategies strengthen the signal for transcripts with high uncertainty. The SEESAW suite of methods is shown to have higher power than other allelic imbalance methods when there is isoform-level allelic imbalance. We also introduce a new test for detecting imbalance that varies across a covariate, such as time.

Background
Genome-wide association studies (GWAS) have identified tens of thousands of genomic loci that are associated with complex traits or diseases, many of which are located in non-coding regulatory regions [1]. One potential mechanism by which allelic variation in these non-coding regions may affect phenotype is that the variants reside in transcription factor (TF) binding sites and influence the activities of TFs and transcription. Such a non-coding region may be referred to as a cis-regulatory element (CRE). Individuals that are heterozygous at such a variant may exhibit imbalanced allelic expression at any genes regulated by the CRE. With RNA-sequencing (RNA-seq) experiments, it is possible to observe such imbalance in allelic expression in the sequenced reads for those individuals also heterozygous for a variant in the exons of a regulated gene; other mechanisms of allelic imbalance, e.g., sequences affecting splicing or post-transcriptional regulation, are also possible to be detected. Recent advances in long-read technologies enable reconstruction of individual diploid genomes/transcriptomes, which leads to more accurate analysis at allele and isoform resolution. Analysis of allelic imbalance (AI) has the potential for higher power to detect cis-genetic regulation than analysis of total expression, as trans-regulatory and non-genetic effects on expression level are controlled for when comparing the...
two alleles within samples [2–8]. Such effects controlled for in AI analysis include both biological variability and technical artifacts that may distort total expression levels across genes. AI is also a powerful analysis to reveal cis-genetic regulation in heterozygous individuals that varies across samples representing different conditions, tissues, spatial contexts, or time periods [9–13].

AI can be isoform-specific, but due to low statistical power and challenges to statistical inference caused by multi-mapping, AI is often measured at the exon-level or gene-level. If different isoforms are subject to regulation from different sets of CRE, and these harbor genetic variants for which the individual is heterozygous, then such isoforms may exhibit different strength or direction of AI, as has been observed recently in an analysis of genomic imprinting at isoform level [14] and in a survey of expression in GTEx using long-read technology [15]. Being able to detect AI at the isoform level could provide insight into mechanisms of complex traits and diseases. One challenge is that AI can only be observed when the individuals under study are heterozygous at an exonic variant. Furthermore, only a subset of reads that can be aligned or probabilistically assigned to a transcript or gene will provide allelic information. As described by Raghupathy et al. [16], an RNA-seq read can fall into various categories of multi-mapping with respect to gene, isoform, and allele, providing different information for expression estimation or “quantification” at different levels. The uncertainty in measuring the expression level from multi-mapping is referred to here as inferential uncertainty. When variants lie in exons that are not constitutive, reads overlapping these exonic variants provide information for isoform-level AI. In the other case, if exonic variants lie in constitutive exons, or in exons of dominant isoforms, AI can be effectively detected by existing methods such as phASER [8] or WASP [17], which count reads aligning to gene haplotypes. WASP for example examines imbalance in the pileup of reads mapped to the genome, after correction for technical bias due to differential mapping rates of the two alleles [18]. WASP can be followed up by methods such as ASEP for statistical inference of gene-level AI across a population of individuals that may be homozygous or heterozygous for regulatory variants [19].

A subset of existing methods are able to detect cis-genetic regulation at sub-gene resolution from short-read RNA-seq. Paired Replicate Analysis of Allelic Differential Splicing Events (PAIRADISE) can extract more information about allelic exon inclusion events, by counting reads that overlap both an informative splice junction and an exonic variant for which a subject is heterozygous [20]. Within the PAIRADISE framework, reads are mapped to personalized genomes based on phased genotypes. PAIRADISE provides a statistical model for detecting allele-specific splicing events, by aggregating allelic exon inclusion within individuals, and builds upon their previous method GLiMMPS to detect splicing quantitative trait loci (QTL) across donors of all genotypes [21]. IDP-ASE combines counts of reads from short-read RNA-seq falling along regions of exons with better resolved isoforms and alleles using long reads [22]. A potential limitation for approaches that count reads overlapping specific regions of genes is that these may not be able to fully aggregate allelic information from paired-end reads overlapping multiple informative features along the length of a transcript. For PAIRADISE, some cases of isoform-level AI may be missed when focusing on reads overlapping splice junctions, such as allele-specific differences in length of 5′ or 3′ untranslated regions (UTR).
Other method publications that have demonstrated quantification of expression at allelic- and isoform-level include EMASE [16], kallisto [23], mmseq [24], and RPVG [14]. EMASE proceeds in a similar manner to PAIRADISE, by first constructing a diploid reference; however in this case, EMASE aligns reads to a diploid transcriptome, constructed via the g2gtools software. The EMASE authors found that hierarchical assignment of reads based on their information content in some cases outperformed equal apportionment as would occur using EM-based algorithms such as RSEM [25], kallisto [23], and Salmon [26] with a diploid reference transcriptome. mmseq allows for alignment of reads to a diploid reference transcriptome using Bowtie [27] and additionally can take into account gene-, isoform-, and allelic-multi-mapping when performing inference across alleles in its mmdiff step [28]. mmdiff computes posterior distributions of expression of each feature via Gibbs sampling. Features can be aggregated at various levels of resolution by summing the posterior expression estimates within each sample. Aggregation also has proved an effective strategy in non-allelic contexts, as demonstrated in tximport [29], SUPPA [30], and txrevise [31]. mmseq also provides a method mmcollapse [28] to perform data-driven aggregation of features to reduce marginal posterior variance, although this procedure cannot currently be combined with differential analysis across alleles (i.e., AI analysis).

We introduce a suite of methods, Statistical Estimation of Allelic Expression using Salmon and Swish (SEESAW), for allelic quantification and inference of AI patterns across samples. With the objective of detecting isoform-level AI, we introduce a strategy to group isoforms based on their transcription start sites (TSS). Through simulation, we show that aggregating isoform-level expression estimates to the TSS level can have higher sensitivity than either gene- or isoform-level analysis. SEESAW utilizes Salmon [26] to estimate expression with respect to an allele-specific reference transcriptome, and a non-parametric test Swish [32] to test for AI. Swish incorporates inferential uncertainty into differential testing and makes no assumption of the distributional model of the data. SEESAW follows the general framework of mmseq and mmdiff for haplotype- and isoform-specific quantification and uncertainty-aware inference. Here, the SEESAW methods were applied to simulated data to benchmark against previously developed methods for detection of AI within heterozygous individuals, making use of multiple individuals as biological replicates. We applied SEESAW to an F1 mouse time course dataset, where it detected genes containing both gene-level AI and isoform-level AI. SEESAW can detect cases of AI that are consistent across all samples, differential AI across two groups of samples, or dynamic AI over a covariate, with a new correlation-based test. The statistical testing in SEESAW is available via the Swish function in the fishpond package [33] including a software vignette for allelic analysis.

Results

SEESAW
We first briefly describe the estimation and statistical testing steps in SEESAW (Fig. 1), which combines both existing and new functionality across a number of software packages, with further details provided in the “Methods” section. SEESAW assumes that phased genotypes are available, and is primarily designed for multiple
replicates or multiple conditions of organisms with the same genotype. This can occur with multiple replicates of an F1 cross, or cell lines from individual human donors across developmental time points [34–36], or across conditions [37–40]. First, g2gtools is used to create a diploid transcriptome, which is indexed by Salmon [26] and used for estimating allele-specific expression with bootstrap replicate datasets to assess inferential uncertainty across genes, isoforms, and alleles (detailed SEESAW pipeline shown in Additional file 1: Fig. S1). This approach for allelic quantification, mapping reads to a custom diploid transcriptome, has been demonstrated as a successful strategy in previous work [16, 23, 24, 41], similarly for mapping reads to a spliced pangenome graph [14] or to a custom diploid genome for allelic read counting [7, 42–45]. Next, SEESAW facilitates importing the estimated allelic counts at various levels of aggregation: no aggregation (labelled hereafter “isoform,” or equivalently “transcript”*/“txp”), transcription start site aggregation (“TSS”), or gene-level aggregation (“gene”). Finally, we leverage the Swish [32] tool for differential expression analysis to test across the two alleles within samples via the Wilcoxon signed rank test statistic, averaging over inferential replicates, and using the qvalue package [46] applied to permuted datasets for defining false discovery rate (FDR) bounded sets of
features. The steps for testing dynamic allelic imbalance are provided in the “Methods” section.

**Simulation**

Simulation of an F1 cross based on the *Drosophila melanogaster* transcriptome was used to assess method performance when the true AI status of each transcript was known. iCOBRA diagrams [47] were used to assess the sensitivity, or true-positive rate (TPR), and the FDR at nominal FDR thresholds of 1%, 5%, and 10%. Sensitivity was assessed per transcript, where detection of AI for a gene-level method was propagated to each of the gene’s expressed isoforms. We used Integrative Genomics Viewer (IGV) [48] to visualize the distribution of HISAT2 [49] aligned reads along the reference genome, after removing allelic-biased multi-mapping reads with WASP [17]. While SEESAW uses reads mapped to the diploid transcriptome with Salmon, examining genome-aligned reads with IGV allowed us to identify examples of reads that contained both allelic- and isoform-level information (Additional file 1: Fig. S2).

Notably, SEESAW with TSS aggregation had the highest overall sensitivity at 5% and 10% nominal FDR, above any of the gene- or isoform-level methods (Fig. 2). The reason behind the higher overall sensitivity can be seen when stratifying by types of AI, as in Fig. 2B; SEESAW with TSS aggregation was able to detect discordant AI on isoforms within a gene that could be masked after aggregation to the gene level. Discordant AI refers to the case where isoforms within a gene have opposite directions of AI, while concordant AI refers to the case where isoforms within a gene have the same direction of AI. Gene-level SEESAW, gene-level *mmdiff*, and WASP had loss
of sensitivity to detect these discordant cases of AI. In addition, SEESAW with TSS aggregation or gene-level aggregation, gene-level mmdiff, and WASP had similar sensitivity at 1% nominal FDR considering both discordant and concordant AI, and these methods had observed FDR for this nominal cutoff in the range of 0–2%.

Gene-level SEESAW, gene-level mmdiff, and WASP had higher sensitivity than SEESAW using TSS aggregation when AI was concordant across all isoforms of a gene. This is expected as aggregation at the appropriate level strengthens the AI signal while reducing inferential uncertainty, so increasing power. For example, SEESAW had the strongest power to detect AI when information about the grouping of transcripts by true AI signal was used to aggregate allelic counts (“oracle” in Fig. 2A). Both SEESAW and mmdiff at the isoform level did not have as high sensitivity as methods that aggregated signal. UpSet diagrams [50] of the sets of transcripts called by each method compared to the true AI transcripts indicated the highest overlap among the gene-level methods and TSS or oracle aggregation (Additional file 1: Fig. S3).

We demonstrated that error control could be lost if we used allelic log fold change (LFC) in the aggregation step as described in the “Methods” section (Additional file 1: Fig. S4). Aggregating transcripts by the LFC is a form of “double dipping” as it makes use of the counts across samples twice: once to determine allelic LFC for aggregation then again to test for allelic imbalance. Such a procedure can lead to loss of error control as described elsewhere [51]. LFC-based p-values did not follow a uniform distribution under the null hypothesis and would lead to increased FDR with increased sample size.

As SEESAW makes use of Salmon for quantification, with inferential uncertainty measured via bootstraps, we evaluated the accuracy of uncertainty estimation using \( n = 10, 20, 30, 50 \) and 100 bootstraps, respectively to define 95% bootstrap intervals for the estimated counts (see the “Methods” section). Coverage was evaluated by comparing intervals to the true, simulated counts. Overall these results indicated that the default of \( n = 30 \) inferential replicates (bootstrap samples) was sufficient for estimation of inferential uncertainty, with coverage rates close to the target 95%. The coverage rate slightly increased with number of inferential replicates, with a range of \( \sim 2\% \). The coverage rate increased more across aggregation level, with a range of up to \( \sim 10\% \) (Additional file 1: Table S1). The increments in coverage rate were less than 0.5% comparing 30 to 50 inferential replicates within each aggregation level and expression bin. The difference in coverage rate was still small when comparing between 30 and 100 inferential replicates, across all three expression bins (Additional file 1: Fig. S5).

We find that 30 inferential replicates established a good compromise between precision of uncertainty information and computation time and storage.

We assessed if the use of inferential uncertainty by Swish resulted in better error control compared to AI analysis with a beta-binomial generalized linear model applied to Salmon estimated counts, without taking into account uncertainty. Swish had better control of the FDR at all levels of aggregation compared to a beta-binomial generalized linear model applied to estimated counts with Benjamini-Hochberg correction of p-values to control the FDR (Additional file 1: Fig. S6). Salmon estimated counts can have high uncertainty particularly across alleles and isoforms, which often have high similarity in terms of sequence. Collapsing isoforms to TSS or gene
level reduces uncertainty, but allelic multi-mapping remains for AI analysis [16]. This motivated our use of allelic testing methods such as \textit{mmdiff} and \textit{Swish} that take into account inferential uncertainty for estimated allelic counts.

We also assessed the performance of \textit{SEESAW} compared to a new inference pipeline from the \textit{WASP} developers, called \textit{WASP2} (Additional file 1: Fig. S7). \textit{WASP2} was equally sensitive as \textit{WASP} in detecting gene-level AI, while it had less sensitivity than \textit{SEESAW} to detect discordant AI signal as expected since it follows a similar approach to \textit{WASP}. While we used the \textit{locfdr} package [52] for multiple test correction for \textit{WASP}, we found Benjamini-Hochberg [53] correction performed well for computing FDR-bounded sets for \textit{WASP2}.

**Osteoblast differentiation time course**

We used \textit{Swish} to test for AI at various levels (gene, isoform, TSS) in a time course experiment of differentiating osteoblasts from an F1 mouse, with C57BL/6J dams crossed with CAST/EiJ sires (see the “Methods” section). Following creation of the diploid reference transcripts and quantification steps of the \textit{SEESAW} pipeline, we first tested for consistent AI across all nine time points (the “global AI test”). While exploring the osteoblast differentiation data, we observed that for isoforms of a gene with TSS that were near each other (within 50 bp), these isoforms often shared similar estimated allelic fold change as calculated with \textit{SEESAW}. To facilitate data visualization, strengthen biological signal, and reduce inferential uncertainty, we grouped any transcripts with TSS within 50 bp of each other (referred to here as “fuzzy TSS groups”, to contrast with strict basepair-resolution TSS grouping). We tested at different levels of resolution: gene level, isoform level, and TSS level. To compare across these levels, we looked at genes in common: a gene was considered significant for global AI at isoform level or TSS group level if at least one isoform or TSS group within the gene was significant (nominal FDR < 5%). ISO-form-level testing for global AI returned the most genes, with 6116 significant genes, followed by gene-level with 5701 genes, and TSS-level grouping with 5573 genes. The majority of genes (4625) were in common across all three levels of resolution (UpSet plot [50] provided in Additional file 1: Fig. S8).

Gene-level aggregation had high overlap with TSS-level indicating that, at least for global AI testing, most of the AI signal was not masked by discordant direction of AI among isoforms within a gene. Among genes displaying global AI under aggregation to the gene level, the TSS groups within those genes often had estimated imbalance in the same direction as the gene imbalance – 97.3% of significant genes had all of their TSS groups with significant AI having the same direction as the gene-level estimate. However, \textit{SEESAW} was able to detect—among the 2.7% remaining genes—interesting examples of genes that had different direction of AI among its isoforms. A complete list of the 134 genes showing these significant and discordant patterns within gene is provided in Additional file 1: Table S2 and in the Zenodo deposition. For example, \textit{Fuca2} exhibited discordant AI with the CAST/EiJ (CAST) allele more highly expressed than C57BL/6J (B6) for one of the two leftmost (more 5’ ) TSS but less expressed than B6 for the rightmost (more 3’ ) TSS, with both TSS groups significant at < 5% FDR (Fig. 3A).

Another gene of the 134 genes with discordant pattern was \textit{Sparc}, the most highly expressed gene at the last time point in the osteoblast differentiation time course.
Sparc is known to be critical for bone development. With fuzzy TSS aggregation and count filtering, Sparc displayed four transcripts groups, where group 5 (ENSMUSG00000018593-5) had positive allelic LFC (CAST > B6) and the other groups had negative allelic LFC (Fig. 4).

We additionally tested for “dynamic AI” using the correlation test implemented in Swish for testing changes in allelic log fold change over a continuous covariate. We again tested at gene level, isoform level, and TSS level. Gene-level dynamic AI testing returned the largest number of significant genes (nominal FDR < 5%): 57 genes displayed dynamic AI at gene level, 49 genes at TSS level, and 23 at isoform level (Additional file 1: Fig. S9). Those significant genes shared across all levels only represented a third of those detected at gene level, where another third were shared only between gene level and TSS level. Thus TSS-level aggregation appeared to help recover signal that would be lost if only testing at the isoform level.

Interestingly, we detected genes such as Rasl11b that had isoform-level AI trending in different directions over time (Fig. 3B, Additional file 1: Figs. S10 and S11). Rasl11b exhibited dynamic AI for two TSS groups, with the CAST allele more lowly expressed than the B6 allele for TSS group “1” from day 2 to day 6, roughly balanced from day 8 to day 10, and finally with CAST/EiJ more highly expressed from day 12 to day 18. The other TSS group “3” had almost the opposite allelic ratio behavior: CAST more highly expressed earlier in time but both alleles tending toward balanced, low expression at the end of the time course. While for Rasl11b this pattern was also significant when testing at the isoform level, other genes such as Calcoco1 demonstrated the advantage of grouping features: Calcoco1 exhibited dynamic AI for two TSS groups, “5” and “6”, which
differed in the direction of change in the imbalance (Additional file 1: Figs. S12 to S14). Here, the $p$-value and $q$-value for TSS group “6” was reduced when aggregating counts from the isoform to TSS-group level.

**Discussion**

Our new suite of methods, **SEESAW**, can be used to obtain allele-specific abundance with bootstrap replicates used to capture inferential uncertainty across genes, isoforms, and alleles, and to perform statistical testing of global or dynamic AI. We propose to aggregate estimates of allelic expression of isoforms by their TSS to increase statistical power in testing for AI that is a result of heterozygous variants in the promoter or in CRE that affect a particular promoter. We introduced two different AI testing procedures: global AI to test for the existence of consistent allelic fold changes across samples, and dynamic AI to test for non-zero correlation between the log allelic fold change and a continuous covariate. **SEESAW** can also be used to test differential AI between two groups, as introduced in Zhu et al. [32], or more complex designs using a general regression framework. Differential AI testing and differential correlation AI testing are

![Figure 4](image-url)
shown as examples in the allelic analysis software vignette. The above tests utilize non-parametric testing, thus making no assumption on the distribution of the data itself. Nonparametric testing had better performance on the simulated data than a standard beta-binomial generalized linear model. In simulation, we demonstrated that **SEE-SAW** on TSS level had the highest sensitivity in the case that AI was discordant within gene, and achieved an FDR that was close to the nominal value at all levels of resolution (gene-, TSS-, or isoform-level testing), implying **SEE-SAW** can maintain error control despite high and heterogeneous levels of uncertainty. **SEE-SAW** at gene level performed comparably to existing methods such as **WASP** and gene-level **mmdiff**. For the osteoblast differentiation experiment, **SEE-SAW** was able to recover some genes with discordant isoform-level AI across all time points and was able to detect genes with isoform-level AI that changed over time in different directions.

Currently, **SEE-SAW** does not support alignment of haplotypes across individuals of different genotype. SNP-based analysis simplifies this problem, but at a loss of information, as evidence of AI may be distributed across multiple exonic variants within a transcript. A newly developed approach **RPVG** [14] maps RNA-seq reads to a spliced pangenome, and then provides haplotype-specific transcript abundance estimates for each individual. It would require further work for the methods presented here to group individuals by their haplotype combinations per gene and perform across-sample inference while accounting for estimation uncertainty using **Swish**. Another limitation of our current approach is that grouping isoforms together based on their TSS reveals shared promoter-based regulation, but may miss isoform-specific AI caused by intronic variation or variation that affects nonsense-mediated-decay. **IDP-ASE** and **PAIRADISE** provide inference on AI of splicing events, and these methods could be considered for detection of these cases. Alternatively, the framework of **SEE-SAW** can be adapted and used with other aggregation rules for different biological purposes, e.g., aggregating isoforms by various splicing events in a manner similar to **SUPPA** or **txrevise**. While **SEE-SAW** can be used at various levels of resolution, from transcript or TSS group up to gene level, if the focus of interest is gene-level AI, we found that **WASP** and **WASP2** were equally sensitive and had good control of false discoveries, using **locfdr** and Benjamini-Hochberg correction, respectively. Additionally, the **ASEP** [19] method can be applied to allelic counts from **WASP** and allows for detection of gene-level AI across a population using a mixture model to account for the unobserved regulatory variants—individuals that are heterozygous for exonic variants may be either homozygous or heterozygous for regulatory variants. The analytical consequences of multiple regulatory SNPs and varying degree of linkage disequilibrium (LD) of these to the exonic SNP, with respect to detection of AI, have been described previously by Xiao and Scott [4].

While here we relied on gene annotation to group together transcripts and reduce inferential uncertainty of allelic expression estimates, another approach would be to use data-driven aggregation methods such as **mmcollapse** and **Terminus** [54]. We were not able to perform differential testing across alleles with **mmdiff** after aggregation with **mmcollapse**. A future direction that may improve performance with the inclusion of **Terminus** in the **SEE-SAW** pipeline would be stratification of different null distributions for test statistics in **Swish** based on aggregation level (transcript, transcript-group, or gene level).
After having detected AI, a natural next step is to try to understand the mechanism of \textit{cis}-genetic regulation. It is possible to associate the AI seen on transcripts or genes with one or more regulatory variants, either through phasing or usage of population-level LD to establish the search space. The list of candidate regulatory SNPs can be further refined by integrating allelic signal within CRE at the epigenomic level, including allelic binding of proteins [42, 55–57], allelic accessibility [58, 59], or allelic methylation [60]. Alternatively, search for altered transcription factor binding motifs can be combined with RNA or protein abundance of potential regulators to winnow down the list of candidate causal regulatory variants [11, 61, 62]. It may also be of interest to detect in which cell types the allelic signal may be strongest or exclusively present, as has been investigated in recent methods for single cell allelic expression or accessibility datasets [63–65]. Finally, we note that a number of methods have shown that AI can be effectively integrated with total expression across individuals of all three genotypes [5, 13, 66–69]. This approach uses more information and so should produce a gain in sensitivity, as well as extending beyond genes harboring exonic variants, which is a limitation for AI-based methods.

\textbf{Conclusions}

Here we present a new suite of methods, called \textit{SEESAW}, for quantifying and testing AI. \textit{SEESAW} offers analysis at various levels of resolution (isoform, TSS, gene-level) and has significantly improved performance compared to existing methods for detecting when there is isoform-level imbalance. \textit{SEESAW} provides statistical testing for global AI (across all samples), dynamic AI (differences along a continuous axis such as time), or differential AI (across groups of samples). The statistical testing in SEESAW is available in an R/Bioconductor package, \textit{fishpond} [33], with an associated software vignette and visualizations designed specifically for allelic analysis.

\textbf{Methods}

\textbf{SEESAW}

The following steps were applied to analyze isoform-, TSS-, or gene-level AI. A sample-specific diploid transcriptome was constructed using \textit{g2gtools} with the following input data: a reference genome (FASTA file); haplotype-specific variants (SNP and indel VCF files); and a catalog of all possible transcripts in the reference genome (GFF or GTF file). \textit{g2gtools} was used to patch and transform the reference genome using the SNPs and indels from the the VCF file, and to extract transcripts from each haplotype of the custom diploid genome. Combined, these transcripts form the custom diploid transcriptome used to quantify RNA-seq reads. \textit{Salmon} [26] was used to quantify expression at the level of allelic transcripts, where both alleles are kept in the reference during indexing (\texttt{--keepDuplicates}). During the quantification step, 30 bootstrap replicates were generated to capture inferential uncertainty across genes, isoforms, and alleles (\texttt{--numBootstraps 30}).

To increase statistical power in testing for AI at sub-gene resolution, we recommend to group isoforms at a resolution that prioritizes discovery of \textit{cis}-genetic regulation effects from non-coding variation in the promoter or in CRE that affect a particular promoter. Aggregation of isoform expression to higher levels has been shown to reduce inferential uncertainty and may improve detection power as long as the signal of interest
is not at the same time lost or diminished through aggregation [28, 29, 54]. Other related approaches include performing inference at the level of equivalence classes (partitions of reads) [70, 71], although here we focus on analysis performed over sets of one or more transcripts for their biological interpretability. During aggregation, both estimated counts (point estimates) and bootstrap replicate counts were summed across isoforms within a TSS-based group for each allele. TSS groups can be defined strictly (identical start position) or with some basepair (bp) tolerance (“fuzzy TSS groups”). After aggregation, every aggregate feature will have a point estimate of abundance as well as a vector representing the bootstrap distribution for each of the two alleles. Likewise, we explored gene-level aggregation, summing across all isoforms for a gene.

To evaluate the extent of loss of error control, we also assessed another aggregation approach: grouping transcripts after having “double dipped” on the expression data for AI analysis [51]. For this test, we first performed AI analysis (described below) on transcript-level estimated allelic counts and then aggregated the transcript-level data within gene based on the sign of the transcript-level allelic log fold change (LFC), referring to this as “LFC-based” aggregation. This is not a recommended aggregation strategy within SESAW, but only used here for evaluation of error control.

In the fishpond Bioconductor package, we used a convenience function makeTx2Tss for generating TSS groups (with an optional parameter to group nearby TSS) and then used importAllelicCounts to import the estimated counts, abundances, and bootstrap replicates, producing a SummarizedExperiment object and leveraging the tximeta package [72, 73]. In the case that there was no read information to distinguish the two alleles, e.g., identical sequence, or no reads covering any sequence differences, Salmon splits the total counts equally among the two alleles, so the estimated allelic fold change was equal to 1. Such features were filtered out of the dataset before differential testing, as demonstrated in the software vignette. Prior to differential testing, features that did not have a minimum count of 10 for three or more samples were filtered out.

Swish [32] was used here to detect AI across biological replicates or conditions while taking into account inferential uncertainty. Swish is a nonparametric method originally designed for isoform-level differential expression that extends the gene-level method SAMseq [74]. We tested for the existence of AI (allelic fold change not equal to 1) for a given feature across all samples by specifying a paired analysis with x="allele", pair="sample", which was referred to as “Global AI testing.” Reviewing the paired-sample method developed in Zhu et al. [32], Swish for global AI used the Wilcoxon signed-rank test [75], where the paired data in this context are the allelic counts for each sample and each bootstrap. The signed-rank test statistic was averaged over bootstraps (in this case, 30 bootstraps), and the allelic labels were swapped to generate a permutation null distribution. The framework of averaging nonparametric test statistics over replicate datasets, followed by permutation-based q-value computation, was derived from SAMseq [74].

We further extended Swish to test for changes in AI along a continuous covariate. We tested for non-zero correlation between the log allelic fold change within paired samples and a continuous covariate by specifying, e.g., x="allele", pair="sample", cov="day", which was referred to as “Dynamic AI testing.” Either Pearson or Spearman correlations can be computed between the pairwise log fold changes and the
continuous covariate, and this statistic was then averaged over bootstrap samples. To stabilize the log fold change, a pseudocount was added to the numerator and denominator of the allelic fold change, with a default value of 5, as has been used previously for bulk RNA-seq [32]. The continuous covariate was then permuted and correlations recalculated to generate a null distribution over all permutations and all features, followed by \( q \)-value computation with the \textit{qvalue} package [46] to obtain the significance of the relationship between the allelic fold change with the continuous covariate. Testing for changes in AI across a categorical covariate was already available in \textit{Swish} as an “interaction test” and is demonstrated in the software vignette on allelic analysis. Additional tests can also be performed, following the \textit{Swish} framework of averaging test statistics over bootstrap replicates and performing permutations to compute the \( q \)-values.

An example of testing for differences in correlation of AI with a continuous covariate across two groups using a general regression framework is demonstrated in the software vignette for allelic analysis, under the heading, “More complex designs”.

For statistical methods designed for comparing gene expression across samples, it is common practice that measures of gene expression are scaled or an offset is included in the model to account for a well-known technical bias: differences in sequencing depth across samples affect the observed or estimated counts and if left unadjusted the across-condition estimates would be biased. Since \textit{SEESAW} focuses on testing differences in expression between the two alleles within the same sample, the sequencing depth bias affected both allelic counts equally, and scaling/offsets were not needed. Thus, this scaling step should not be performed.

A number of plotting functions in \textit{fishpond} were used to facilitate visualization of allelic expression changes across samples, isoforms, and covariates. \texttt{plotInfReps} was used to visualize allelic expression estimates and inferential uncertainty across samples and conditions/time points [32]. \texttt{plotAllelicGene} was used to visualize isoform- or TSS-group-level allelic expression data along with a diagram of a given gene model, using the \textit{Gviz} package [76]. \texttt{plotAllelicHeatmap} was used to visualize allelic expression across isoforms or TSS groups and samples, leveraging the \textit{pheatmap} package [77]. In \textit{SEESAW}, transcript ranges were represented using \texttt{GRanges} [78] objects generated from \texttt{TxDb} or \texttt{EnsDb} databases [79] and attached as \texttt{rowRanges} to the main dataset object with estimated counts, abundance and bootstrap counts, facilitating downstream plotting and data exploration.

**Simulation**

To assess the performance of different methods in recovering gene-level and isoform-level AI, we simulated RNA-seq reads from a diploid transcriptome derived from the \textit{Drosophila melanogaster} reference transcriptome, restricted to chromosomes 2, 3, 4, and X, simulating RNA transcripts from female flies. The simulation contained a total of 10 samples, with an average sequencing depth of 50 million paired-end reads per sample. The maternal reference transcripts included the protein-coding and non-coding RNA from Ensembl [80] (release 100), and the paternal reference transcripts were created from maternal transcripts by adding single nucleotide variants. To create paternal alleles, we randomly selected 5 exons from each gene, and the mid-position nucleotides of the selected exons were substituted with their complement nucleotides. Genes
overlapping simple tandem repeats of size 50 bp or larger were excluded from the simulation, leaving 14,821 genes.

While the majority of genes (93.3%) were simulated to not have AI, two types of AI were simulated: “concordant AI,” where all isoforms had concordant allelic fold change, and “discordant AI,” where there were discordant allelic fold changes among the isoforms of the gene. In the discordant AI case, the RNA abundance was balanced across the two alleles when summed across all the isoforms of the gene. We randomly selected 1000 genes using the following criterion: (1) the number of annotated isoforms in the selected gene was between three and six, (2) the gene had at least two isoforms sharing the same transcription start site (TSS), and (3) the gene had at least two distinctive TSS. Half of the selected genes (500) were simulated to have concordant AI and the other half (500) were simulated to have discordant AI, and the remaining 13,821 genes were simulated to have allelic balance.

The abundance of the maternal allele was set to a constant value, and the paternal allelic abundance was altered to generate AI. For genes with concordant AI, the paternal allele was either 25% upregulated or downregulated, chosen at random per gene. Within each gene with discordant isoform-level AI, one TSS was randomly chosen and isoforms sharing the selected TSS had abundance increased on the paternal allele. Abundance was increased such that the upregulation fold change was equal to \(1 + \frac{1}{2n}\), where \(n\) is the number of isoforms with the selected TSS. The other isoforms of the gene had paternal abundance decreased at an equal rate such that the gene-level abundance was kept constant. Expected count values were then generated from the alleles of all transcripts by multiplying abundance by the transcript length and scaling up to the desired library size. Reads were generated using \textit{polyester} [81], with the following settings: fragments with a mean size of 400 bp, paired-end reads of 150 bp, and Negative Binomial dispersion parameter \textit{size}=100, such that the simulation contained across-sample biological variation on the allelic counts and the allelic fold change. Paired-end reads were shuffled so that the reads were listed in a random order. Read generation and all subsequent analysis steps for \textit{SEESAW} and other methods were automated using a \textit{Snakemake} workflow [82] available at the \textit{ase-sim} repository [83].

Additional calculations were performed to evaluate the number of inferential replicates needed to capture inferential uncertainty. The calculation was performed on three levels of aggregation: isoform level, TSS level, and gene level, and across \(n=10, 20, 30, 50\) and \(100\), the number of inferential replicates. Within each aggregation level, features were divided into three expression bins (low/medium/high tertiles) based on true, simulated counts. 95% bootstrap intervals were computed using the bootstrap mean and plus or minus the bootstrap standard deviation multiplied by the 97.5% quantile of the normal distribution. Mean and standard deviation of the rate of interval coverage were calculated across samples, within each aggregation level and expression bin.

A beta-binomial generalized linear model was fit to the same \textit{Salmon} estimated allelic counts as provided to \textit{Swish}, in order to compare the performance of \textit{Swish} to a method that does not make use of information about the uncertainty in quantification. Maximum likelihood estimates of over-dispersion and generalized linear model coefficients were computed using \textit{apeglm} [84] without coefficient shrinkage, and \(p\)-values computed
from the Wald statistics for the allelic log odds ratio. $p$-values were corrected for multiple testing using the Benjamini-Hochberg method [53].

We applied the SEESAW pipeline as well as existing methods mmseq and WASP to the simulated data. As our interest in this work was in identifying isoforms affected by cis-genetic regulation, we focused our comparisons on methods that aim to aggregate allelic information along the entire extent of the transcribed region, whereas other existing methods have focused on allelic differences in internal splice events (splicing quantitative trait loci, or sQTL); we chose a method that attempts to resolve AI at the isoform level (mmseq), as well as a method that resolves bias from genomic multi-mapping reads (WASP) and is primarily focused on gene-level AI. SEESAW and other methods were provided with the complete exonic sequence of the two alleles, and the gene annotation, either as FASTA (for Salmon and mmseq) or VCF files with known phasing (for WASP). We also utilized the ground truth of simulation to obtain an optimal isoform-grouping strategy, called “oracle.” For “oracle” grouping, upregulated or downregulated isoforms within a gene were grouped in cases of discordant AI. Otherwise, all isoforms within a gene were grouped together.

The following steps were used to apply mmseq (version 1.0.10a) and its differential testing step mmdiff to the simulated data. Bowtie (version 1.3.1) [27] was used to index the diploid reference transcripts and to align the reads. During the alignment, only the alignments that fell into the best stratum were reported if the alignments fell into multiple stratum using --best --strata. If more than 100 reportable alignments existed for a particular read, then all alignments were suppressed using the -m option. After obtaining mmseq expression estimates at gene and isoform level, we manually separated the estimates for the maternal and paternal alleles and subsequently used mmdiff to test for differential expression between the two alleles, using the flags -de 10 10 <maternal files> <paternal files>. Posterior probability of equal expression was used to threshold and define significant sets of transcripts or genes.

We ran WASP on the simulated data according to its recommended usage. First, HISAT2 (version 2.2.1) [49] was used to align reads to the bdgp6 reference genome, downloaded from the HISAT2 website. An h5 database was created from the simulation VCF file containing the location of the exonic SNPs and the known phasing information. HISAT2 was used to re-align the reads with flipped nucleotides, and genomic multi-mapping reads that would otherwise bias allelic ratios were filtered. Read counts and heterozygous probabilities were adjusted using WASP scripts. Finally the Combined Haplotype Test (CHT) [17] was applied with recommended defaults --min_counts 50 and --min_as_counts 10 to generate a $p$-value per gene for AI across samples. Multiple testing was controlled via the locfdr package [52], applied to z-scores derived from WASP $p$-value output.

To visually compare the AI simulation results across methods, we used the iCOBRA [85] Bioconductor package. We assessed the performance according to the true and reported allelic status of the transcripts (balanced or imbalanced), where reported significance of AI of a gene or TSS group was propagated to its isoforms. As the simulation consisted only of genes in which all isoforms or no isoforms exhibited true AI, this approach to compare methods at the transcript level should not unfairly impact the performance of the aggregated (gene- or TSS-level) AI tests.
Osteoblast differentiation time course
We applied SEESAW to an RNA-seq dataset of primary mouse osteoblasts undergoing differentiation, from F1 C57BL/6J x CAST/EiJ (B6xCAST) mice, to assess both global and dynamic AI. To obtain the B6xCAST F1 animals, pOBCol3.6GFptpz transgenic dams [86] were bred with CAST/EiJ (JAX Stock number 000928) sires. Pre-osteoblasts like cells were collected from the parietal bones of neonatal mice as previously described [86]. The B6xCAST cultures were conducted at the same time, using the same lots of Fetal Bovine Serum to avoid batch effects due to culture conditions. The pOBCol3.6GFptpz transgenic dams possess the pOBCol3.6GFptpz allele on a C57BL/6J background (> 10 generations of backcrossing). For the culture conditions, collection of RNA and transcriptomic sequencing was conducted as previously described [87].

In the differentiation experiment, which has been described previously [88, 89], pre-osteoblast-like cells were extracted from neonatal calvaria, and cells were FACS sorted based on expression of CFP, as driven by the osteoblast Col3.6 promoter. Differentiation was induced with an osteoblast differentiation cocktail in sorted cells and RNA was collected every 2 days from day 2 to day 18 post differentiation (nine time points). Three technical replicates per time point were combined and quantified together as one biological replicate, after quality checking with FASTQC and MultiQC [90]. Expression data for osteoblasts from C57BL/6J mice of the same experiment are publicly available on the Gene Expression Omnibus at accession GSE54461 [91].

Reference transcripts were generated via g2gtools using a reference genome, strain-specific VCF files, and the reference gene annotation. The GRCh38 primary assembly for Mus musculus was downloaded from Ensembl (release 102) [80], and strain-specific VCF files CAST_EiJ.mgp.v5.snps.dbSNP142.vcf (SNP) and CAST_EiJ.mgp.v5.indels.dbSNP142.normed.vcf (indel) for mm10 were downloaded from the Mouse Genomes Project [92]. Reference transcripts (Mus_musculus.GRCm38.102.gtf) were downloaded from Ensembl (release 102) [80] and subsequently were patched and transformed for the CAST/EiJ strain using g2gtools (version 0.2.7). All code including a Snakemake workflow [82] for generation of the diploid transcriptome is provided in the diploid_txomes directory of the osteoblast-quant repository [93].

Salmon was used to quantify the RNA-seq reads against the custom diploid transcriptome with 30 bootstrap replicates, and these data were imported into Bioconductor and analyzed with Swish as described in the “SEESAW” section above. Swish with global AI test was performed on isoform level, TSS-group level, and gene level, and results were compared at various levels of resolution. In addition, we used the newly developed feature in Swish to test for dynamic AI: we tested the correlation between the log fold change comparing across alleles within a sample and the day of differentiation, using the Pearson correlation.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13059-023-03003-x.

Additional file 1: Supplementary figures and tables. Additional file 1 includes 14 figures and 2 tables. The supplementary figures describe the pipeline of SEESAW, details on both simulated and osteoblast datasets, as well as
benchmark results with existing methods. The tables display bootstrap coverage and the list of genes with discordant AI in the mouse F1 time course.

Additional file 2. Review history.

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Peer review information

Andrew Cosgrove was the primary editor of this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Review history

The review history is available as Additional file 2.

Authors' contributions

EYW, NPS, KC, RP, and MIL conceived of the quantification and statistical analysis methods. EYW and MIL wrote the new code and documentation for fishpond. EYW, NPS, RP, and MIL developed the simulation framework. EYW and MIL analyzed the performance of methods in simulation. KC, MV, GAC, and CLA-B provided data and expertise for analysis of the mouse F1 cross dataset. EYW analyzed the mouse F1 dataset and generated results tables and diagrams. EYW, NPS, KC, MZ, and MIL made major contributions to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

SEESAW is available in the fishpond [33] Bioconductor package, distributed with a GPL-2 open source license. Version 2.4.1 of fishpond was used in this manuscript, available from Bioconductor (release 3.16) Bioconductor [94]. The allelic analysis software vignette can be viewed at the package website. Code for quantifying the osteoblast data (using Salmon version 1.5.2) is provided in the osteblast-quant repository [93] and code for running Swish and compiling the results from other methods is provided at the swish-ase-assessment repository [96].

The B6xCAST mouse osteoblast RNA-seq experiments are available at the SRA under project accession SRP036025 [97]. For the mouse osteoblast dataset, R data objects containing the total and allelic counts at gene and isoform level, diploid transcriptome sequences, Salmon quantification directories for all samples, global and dynamic AI test results at all three levels of resolution, and the discordant global AI gene list are provided on Zenodo [98]. For the Drosophila melanogaster simulated samples, R data objects containing the simulated abundances and status of each transcript, simulated transcriptome sequences, Salmon quantification directories for all samples, SummarizedExperiment objects at various levels of resolution, and results tables for each method are provided on Zenodo [99].

Declarations

Ethics approval and consent to participate

All animals were raised at The Jackson Laboratory. Mouse husbandry and all experiments were performed in accordance with The Jackson Laboratory Institutional Animal Care and Use Committee-approved protocols (Animal Care and Use Committee, ACUC).

Consent for publication

Not applicable.

Competing interests

RP is a co-founder of Ocean Genomics Inc.

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