Table 10. Validation of selected genes from study comparisons by qRT–PCR

Name	Transcript ID	qRT–PCR FC	Corbo FC	Mustafi FC	This report FC	p-value	WT FPKM	Nrl$^{-/-}$ FPKM
Corbo								
Abca13 exon 53/55	NM_178259	NA	−11.58	NA	−4.18	0.000278	0.75	0.18
Abca13 exon 56/58	NM_178259	−21.36	−11.58	NA	−4.18	0.000278	0.75	0.18
Amz2	NM_025275	1.09	−6.77	1.13	1.06	0.1122	15.06	16.13
Klf9	NM_010638	1.09	−2.6	−1.15	1.11	0.2481	29.7	33.12
Pip5k1a	NM_008847	1.43	50	1.03	1.35	0.0015	11.81	16.25
Sema7a	NM_011352	1.03	20	−1.39	1.13	0.0634	13.33	15.19
Txnip	NM_023719	−1.07	12.5	1	1.04	0.7347	2.17	2.01
Mustafi								
Cox5b	NM_009942	1.26	NA	−12.15	1.01	0.9342	18.31	18.24
Drd4	NM_007878	−1.23	NA	−8.72	−1.92	0.0764	39.6	20.92
Cd8a	NM_009857	11.5	NA	21.17	9.03	3.47E-06	0.08	0.73
Ctss	NM_021281	1.25	NA	6.92	1.29	0.0228	3.82	5.23
Corbo-Mustafi								
Acoxl	NM_028765	−28.6	−13.93	−34	−4.61	0.0291	0.28	0.06
Rpgrip1	NM_001168515	−1.3	−2.32	−1.83	1.06	0.1157	29.94	31.85
Dynlt3	NM_025975	1.28	5	3.45	1.46	0.0036	17.79	26.39
Rab18	NM_181070	1.37	3.23	3.31	1.29	0.0028	33.71	43.77
Neurod1	NM_010894	1.38	2.63	2.2	1.14	0.0888	168.51	192.86
This report								
Plekhf2	NM_175175	−5.88	NA	−1.35	−5.35	0.000428	37.77	7.21
Klhl3	NM_001195075	−6.9	NA	NA	−3.29	0.000101	8.62	2.6
Ccdc24	NM_001034876	−2.93	NA	NA	−2.64	0.0031	66.86	24.85
Rgs22	NM_001195748	11.24	NA	NA	3.84	5.93E-07	1.95	7.47
Hr	NM_021877	3.82	NA	1.25	3.89	0.000307	16.38	63.51
Wscd2	NM_177292	4.1	NA	1.13	4	0.000123	5.73	22.91
Klhl33	NM_001166651	27.12	NA	NA	14.03	2.34E-06	3.02	42.25

Differential expression of genes identified in three global profiling studies (Corbo, Mustafi and this report) was validated by qRT–PCR. Genes shown under the Corbo and Mustafi subheadings were identified in respective studies, but not in the current study. Corbo-Mustafi subheading indicates genes identified in both Corbo and Mustafi studies but not in the current report. The two genes, *Cd8a* and *Acoxl,* identified previously were differentially expressed by qRT–PCR but were filtered out of our sequencing data because of the low FPKM values observed. The gene *Abca13* was detected as only expressing the last 7 exons of the annotated sequence. qRT–PCR assays that distinguished between the two isoforms were able to confirm the differential expression of the last 7 *Abca13* exons. This gene was filtered out of our sequencing results because of the low FPKM value observed. All genes included in this report subheading were uniquely identified by the current study (and not in Corbo and Mustafi reports). FC, p-val, and FPKM values in this report are derived from the sequencing results reported here. Genes were selected as having the largest fold changes in each category and from current annotation in the latest mm9 Refseq build. All differentially expressed genes identified in this report could be validated by qRT–PCR. FC=fold change, NA=not applicable, FPKM=fragments per kilobase exon model per million reads.

alternate splicing. The higher accuracy of quantitative gene expression estimates by the BWA workflow compared to those by TopHat is evident from the stronger correlation determined by linear regression analysis of the DETs. The regression line from BWA had a slope of −1.056 (compared to −0.905 for TopHat) and R^2 of 0.8798 (compared to 0.7727 for TopHat).

The TopHat workflow maps the reads to exonic regions of the reference genome as well as across all known and putative splice junctions defined in the Ensembl GTF file. TopHat attempts to map reads across splice junctions defined in the Ensembl GTF file and across novel splice junctions detected during the first phase of alignment. Hence, the TopHat workflow maps significantly more reads starting with the same number of pass filter (PF) reads and detects additional transcript isoforms missed by the BWA workflow. The source of genomic annotations used by these methods is another important difference. UCSC refFlat annotation (used by the BWA workflow) for the mouse reference genome (build mm9) contained approximately 28,000 unique transcript isoforms, whereas the Ensembl GTF file (used by the TopHat workflow) for the same genome build listed three times more unique transcript isoforms. The problem is amplified because of the lack of one-to-one mapping for several transcripts defined in the UCSC refFlat file in Ensembl GTF. Hence, a non-trivial number of DETs detected by the BWA workflow could not be mapped to any DET from the TopHat workflow (see Figure 2, regions shaded in green).

The BWA workflow detects about 16,000 transcripts in the retina, with a minimum expression equivalent to one transcript per cell (i.e., 1 FPKM) [16]. When the criteria were relaxed to cover transcripts expressed at low levels (0.1 FPKM), 20,707 transcripts were detected in the retina. This is not surprising as the whole retina includes more than 50 distinct neuronal cell types, and each cell would achieve protein diversity largely by alternative promoter usage and/or alternative splicing [68]. The TopHat workflow yields thousands of known and putative transcript isoforms not previously described in the retina. However, validating these novel isoforms predicted from RNA-seq data remains a challenge.

Integrated analysis of RNA-seq data with miRNA-seq, transcription factor binding sites data (chromatin immunoprecipitation sequencing-Chip-Seq), genetic variations (expression Quantitative Trait Loci) [69], and methylation patterns would allow decoding of the complex regulatory networks associated with retinal development and function. Several technical improvements would however be necessary to overcome the bias introduced into the RNA-seq data due to GC content, mappability of reads, length of the gene, and regional differences due to local sequence structure [70]. RNA-seq methods are more likely to identify longer differentially expressed transcripts than shorter transcripts with the same effect size [71]. New statistical methods are being developed to correct for systematic biases inherent in NGS data [70-72]. In the coming years, we will witness an explosion in high throughput genomic methods that are expected to revolutionize biology and biomedical discovery.

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