Characterizing the genetic basis of transcriptome diversity through RNA-sequencing of 922 individuals

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Abstract

Understanding the consequences of regulatory variation in the human genome remains a major challenge, with important implications for understanding gene regulation and interpreting the many disease-risk variants that fall outside of protein-coding regions. Here, we provide a direct window into the regulatory consequences of genetic variation by sequencing RNA from 922 genotyped individuals. We present a comprehensive description of the distribution of regulatory variation – by the specific expression phenotypes altered, the properties of affected genes, and the genomic characteristics of regulatory variants. We detect variants influencing expression of over ten thousand genes, and through the enhanced resolution offered by RNA-sequencing, for the first time we identify thousands of variants associated with specific phenotypes including splicing and allelic expression. Evaluating the effects of both long-range intra-chromosomal and *trans* (cross-chromosomal) regulation, we observe modularity in the regulatory network, with three-dimensional chromosomal configuration playing a particular role in regulatory modules within each chromosome. We also observe a significant depletion of regulatory variants affecting central and critical genes, along with a trend of reduced effect sizes as variant frequency increases, providing evidence that purifying selection and buffering have limited the deleterious impact of regulatory variation on the cell. Further, generalizing beyond observed variants, we have analyzed the genomic properties of variants associated with expression and splicing, and developed a Bayesian model to predict regulatory consequences of genetic variants, applicable to the interpretation of individual genomes and disease studies. Together, these results represent a critical step toward characterizing the complete landscape of human regulatory variation.

Introduction

Unraveling the genetics of human gene expression and describing the landscape of genetic variants affecting the transcriptome will reveal important insights into the architecture and control of the human regulatory network and allow us to more fully characterize the non-coding, regulatory regions of the genome. Population-level studies of gene expression combined with genotyping allow us to directly evaluate the association of genetic variation with expression (Goring et al. 2007; Stranger et al. 2007), revealing expression quantitative trait loci (eQTLs) in a variety of populations, tissues, and contexts (Dimas et al. 2009; Grundberg et al. 2012; Stranger et al. 2012; Liang et al. 2013). Many genetic variants shown to have impact on expression also affect higher-level traits including disease risk (Emilsson et al. 2008; Nica et al. 2010; Fairfax et al. 2012), and through investigation of expression as a cellular phenotype, we can provide a more mechanistic interpretation of individual functional variants. Further, with the advent of RNA-sequencing technology, we are now able to assay the complete transcriptome, providing access to a wider range of expression traits, including distinct isoforms and allelic expression (Mortazavi et al. 2008; Wang et al. 2008; Trapnell et al. 2010). Initial studies in cohorts of 60-70 individuals have combined RNA-sequencing and genetic information to identify variants with impact on this broad range of transcriptional phenotypes (Montgomery et al. 2010; Pickrell et al. 2010) but were limited in power and sequencing depth to fully describe the impact at the regulatory network and genome levels.

Here, we leverage the resolution offered by RNA-sequencing in a large population study utilizing a primary human tissue. We have sequenced RNA from whole blood of 922 genotyped individuals from the Depression Genes and Networks cohort (Methods), all of

European ancestry. Here, we describe the impact of local and distal regulatory genetic variation on diverse expression traits, characterizing the distribution of QTLs according to the specific expression phenotypes altered, the properties of affected genes, and the genomic characteristics of regulatory variants.

We find evidence for widespread impact of genetic variation on transcriptional phenotypes of over ten thousand genes, including variants affecting total gene expression, alternative splicing, and allelic expression. We specifically increase the number of known splicing QTLs by nearly an order of magnitude. By evaluating distal, genome-wide regulatory impact of each genetic variant, we highlight a pattern of modularity, or co-regulation of many genes by a smaller number of individual genetic variants, and intra-chromosomal modules specifically influenced by the complex three-dimensional configuration of each chromosome in the nucleus. Further, by analyzing the genes affected by regulatory variants, we find evidence consistent with effects of selection and buffering to limit the downstream, potentially harmful consequences of regulatory variation. Specifically, important genes, including hubs in protein-protein interaction networks, transcription factors, and highly conserved genes are each depleted for *cis*-eQTLs. Finally, utilizing the sizeable set of detected regulatory variants, we characterize the genomic properties of variants affecting both total expression levels and gene splicing, and present a Bayesian framework, the Latent Regulatory Variant Model (LRVM), that combines these diverse properties into a unified model to predict the consequences of genetic variation. This framework offers a method for automatically evaluating the potential regulatory impact of genetic variants observed in future studies.

Results

The Depression Genes and Networks cohort is comprised of individuals of European ancestry within the United States. RNA was extracted from whole blood and, following globin RNA reduction (GLOBINclear^{TM} kit (Invitrogen)), sequenced in an Illumina HiSeq 2000, yielding approximately seventy million 51bp single-ended reads per individual. After quality control, 922 individuals remained for analysis in this study (Figure S2-S4). Each individual was also genotyped for 720,591 autosomal single nucleotide polymorphisms (SNPs) on the Illumina HumanOmni1-Quad BeadChip (Figure S1). For details of sample collection and data processing, see Methods and Supplementary Materials.

From these data, we identify expression quantitative trait loci (eQTLs) affecting the large majority (78.8% at FDR 0.05) of genes with quantifiable expression (Methods, Table 1, Data S1). Notably, despite evaluating expression in a single tissue, 680 known trait- and disease-associated variants, out of 1,445 tested (Hindorff et al. 2009) (see Methods), are associated with expression phenotypes in this study, including 159 loci specifically associated with gene splicing (Table S2, S3). In each class of QTL, particularly among *trans* associations and disease variants, the large sample size was essential to identifying the full range of regulatory effects (Figure S10).

Prevalence and impact of proximal regulatory variation.

We find broad impact from proximal regulatory variation across the genome including *cis*-regulatory variants associated with total gene expression for 10,914 out of 13,857 autosomal genes evaluated (SNPs within 1Mb of the transcription start site, FDR 0.05). Further, while common genetic variants explain a small portion of total expression variation in each gene, their effect is greater than that of significant demographic factors, with *cis*-eQTLs explaining a median of 3.3% of expression variance (median 7.7%

among genes with an eQTL), compared to 0.7% explained by age and sex combined (Figure S12, Table S3). We do not detect substantial modulation of *cis*-eQTLs by sex or age, but considering strong correlation between demographic variables and other covariates including medication and depression status in this cohort, these effects warrant further investigation. We observe high replication between our reported *cis*-eQTLs and large microarray studies (Fehrmann et al. 2011; Grundberg et al. 2012) (ranging from 51% to 89% replication rate, Supplementary Material), though we do identify additional eQTLs with weaker association strength and among lower-expressed genes (Figure S11), suggesting that statistical power along with the resolution of RNA-seq is a contributor to finding eQTLs unique to this study. Genetic variants near each gene also affect alternative splicing; using isoform ratio (the fraction of a gene's expression arising from each isoform, Methods) as a quantitative trait, we identify 2,851 transcripts from 1,370 unique genes with splicing QTLs (sQTLs) at FDR 0.05. While our results agree with previous studies indicating less variation in gene splicing than in total expression (Gonzalez-Porta et al. 2012), we increase the number of splicing variants by nearly an order of magnitude beyond previous studies, which identified up to 324 transcripts affected by genetic variation at FDR 0.05 (Kwan et al. 2008; Pickrell et al. 2010). Again, genotype explains much more variance in isoform ratio than do age and sex – although most isoforms have no significant association, twice as many isoforms have a genetic association than have a demographic one (Figure S12). Furthermore, as hundreds of disease-associated loci are found among the identified proximal regulatory variants, this increased compendium of splicing effects offers new potential to elucidate functional mechanisms. In our analysis, for instance, *rs3865444*, a SNP associated with Alzheimer's disease (Hindorff et al. 2009; Naj et al. 2011), has a strong association with isoform ratio of *CD33* ($p < 1e-200$), far stronger than its association with total expression (p < 1e-20), suggesting a specific regulatory mechanism not previously considered for this variant.

Allele-specific expression (ASE) provides a more detailed evaluation of the distribution of *cis* effects – individuals who are heterozygous for a *cis*-regulatory variant will demonstrate allelic imbalance where one homologous copy of a gene is more highly expressed than the other copy (Yan et al. 2002; Pastinen and Hudson 2004). Here, from ASE evaluated both per-individual and across the cohort, we identify shared genetic drivers of allelic imbalance, and additionally estimate the relative frequency of common and rare *cis* regulatory effects. First, in a novel analysis, we identify a set of regulatory variants that are consistently associated with allelic imbalance in nearby genes across our cohort, where previous studies either identify only instances of ASE per individual without identifying the associated regulatory variants, or require specialized assays measuring allelic expression (Serre et al. 2008; Ge et al. 2009). RNA-sequencing enables direct quantification of ASE (Verlaan et al. 2009; Zhang et al. 2009; Montgomery et al. 2010; Pickrell et al. 2010), and leveraging the number of heterozygous individuals available here, we treat ASE as a quantitative trait itself and are able to identify *specific* genetic drivers of allelic expression. We evaluate *aseQTLs* by testing for statistically significant associations between heterozygous status at individual regulatory variants and allelic imbalance at nearby expressed coding loci, tested exclusively among heterozygous individuals at each coding locus (Figure 1a,b, Methods). The resulting aseQTL associations provide strong evidence of *cis*-regulatory impact and are less likely than standard *cis*-eQTLs to arise from non-*cis* mechanisms, artifacts, or linkage of a non-causal SNP with a rare variant. As expected, analysis of aseQTLs demonstrates an enrichment of allele-specific effects among *cis*-eQTLs (Figure S13-S15). While the power to detect aseQTLs in this study is still constrained by availability of heterozygous individuals and read depth (Figure S16), we are able to directly confirm that 641 of our *cis*-eQTL SNPs are also associated with changes in allele-specific expression in the corresponding gene at FDR 0.05, providing a sizeable catalog of high-confidence *cis*-acting variants. This analysis also indicates a moderately high replication rate (50-70%, Figure S16c) among *cis*-eQTLs with sufficient power (at least 200 heterozygous individuals for varying read depths). Further, 936 exonic loci (in 528 genes) have globally significant aseQTL associations. The strongest aseQTL association is found between heterozygosity at *rs4950928* and allelic imbalance within *CHI3L1* (p < 1e-71, Figure 1b). Previous studies have demonstrated associations for *rs4950928* with asthma and serum levels of *CHI3L1* (Ober et al. 2008), which the aseQTL result supplements with strong evidence for a direct *cis*-acting regulatory effect of this disease variant.

In addition to evaluating genetic drivers of allelic expression shared across many individuals in the cohort, we also evaluate significant ASE events in each individual (Supplementary Materials) in order to assess the distribution of both common and rare regulatory effects. We find that most instances of ASE in each individual can potentially be explained by a common regulatory variant, specifically an eQTL SNP identified in the population (Figure 1c), with 73.8% of individual ASE events co-occurring with heterozygous status for the single best *cis*-eQTL SNP of the same gene. However, there are many cases of ASE (21%) not co-occurring with any explanatory eQTL SNP, and such instances are candidates for rare regulatory variation in that individual – while ASE could arise from non-genetic factors such as allele-specific methylation or imprinting, previous studies suggest genetic variation is the primary cause of ASE (Zhang et al. 2009). Overall, analysis of allelic expression provides direct evidence of *cis* mechanism for a large set of variants (aseQTLs), and additionally allows us to compare the distributions of common regulatory variation with other regulatory factors that would include rare genetic variation, with evidence here suggesting that common genetic variants are responsible for more transcriptional variation.

Distant variants and modular regulation.

In order to address the impact of genetic variation on the full transcriptional network, we also evaluate distal regulatory relationships, both within and across chromosomes. First, the impact of genetic variation even through *cis*-mechanisms is not limited to a small range as measured by genomic distance. Enhancers are known to act over distances up to several Mb away (Nobrega et al. 2003; Kleinjan and van Heyningen 2005), and 3-D configuration of chromosomes within the nucleus can alter the true spatial proximity of regulatory sequences with potential target genes (Spilianakis and Flavell 2004; Lieberman-Aiden et al. 2009; Cheung et al. 2010). We performed a separate, stepwise association analysis to identify *all* regulatory variation influencing genes within each chromosome (Methods), allowing multiple independent eQTLs per gene. Consideration of this expanded set of SNPs for each gene sacrifices some statistical power (we would identify 8,580 eQTL genes at FDR 0.05), but we identify 381 genes affected by SNPs more than 500kb away from the transcription start site (TSS), including 269 genes affected by SNPs over 1Mb away (Table 1). An analysis of allelic effects provides evidence that many variants well outside the standard promoter region still act through *cis*-mechanisms (Figure 2a), though others could arise from indirect *trans* events such as a co-location of a *cis*-regulated transcription factor with a number of its target genes on the same chromosome.

The chromosome-wide analysis reveals a pattern of *modules* of co-regulated genes, with 803 eQTL variants affecting two or more genes and 106 variants affecting three or more.

Co-expression of nearby genes has been reported for several organisms (Lercher et al. 2002; Oliver and Misteli 2005), and our analysis specifically detects genetic variation that affects modular co-regulation, and thus points to genomic regions with regulatory impact on groups of genes. Indeed, the co-regulated genes here are co-expressed in excess of other proximal gene pairs ($p < 1e-87$) and share direction of eQTL effect (Figure S17). These results provide a much larger number of genetically co-regulated genes than previously known, but indicate a similar rate of shared regulation, with 11% of eQTL genes compared to 8% reported previously (Stranger et al. 2012). On the other hand, among the 1,556 genes in any of these modules, nearly half (769) also have a gene-specific (targeted) regulatory variant. An example of both coordinated and targeted regulation affecting five co-located genes (*ADCY7*, *BRD7*, *NKD1*, *NOD2*, and *CYLD*) is shown in Figure 2b, S18. Such clusters tend to occur in gene-dense spatially co-located regions of each chromosome ($p < 1e-47$, Supplementary Materials). Beyond linear proximity among co-regulated genes, three-dimensional chromosomal configuration influences regulatory modules. Specifically, falling within the same topological domain derived from Hi-C chromatin interaction frequencies (Dixon et al. 2012) is predictive of co-regulation by the same variant (log-odds > 0.75 Figure S19, Supplementary Materials). Together these results indicate that coordinated regulation of three-dimensionally co-located genes is common, particularly in gene-rich regions, but even genes with shared eQTLs are also often under the control of specific, targeted regulatory elements.

Furthermore, analyses of regulatory variants that affect expression *across* chromosomes (*trans*-eQTLs) allow us to more fully characterize the structure of the regulatory networks that govern expression traits genome-wide. After applying stringent filters to account for spurious associations arising among regions of sequence similarity (Methods, Supplementary Materials, Figure S20), we identify a set of 138 genes whose expression is associated with a distant SNP and, interestingly, five *trans*-QTLs affecting isoform ratios rather than total expression for three genes. The proportion of *trans* splicing QTLs (compared to both proximal and distant sQTLs) is lower than that of expression QTLs, suggesting that splicing is disproportionately affected through *cis* mechanisms. As with the intra-chromosomal associations, we observe *modularity* among the *trans* associations, with 20% of associated SNPs affecting two or more genes, helping to identify genomic regions that coordinate the regulation of multiple genes (Emilsson et al. 2008; Gilad et al. 2008; Fehrmann et al. 2011). The largest module is a set of 57 genes, enriched for platelet aggregation function (p < 1e-7), all affected by the SNP *rs1354034*, previously associated with mean platelet volume (Fehrmann et al. 2011; Gieger et al. 2011). Additionally, the majority of *trans*-eQTLs SNPs (76 of 138) also have *cis*-regulatory effects, affecting proximal gene expression levels (74 of 76) or isoform ratio (31 of 76, a higher fraction than expected from the global prevalence of sQTLs, p < .05) (Figure S21). One example, *rs10251980*, is a *cis*-eQTL for *IKZF1*, whose loss of function has been linked to prognosis in leukemia (Mullighan et al. 2009). The SNP affects eight distant genes, five of which are up-regulated in response to tretinoin treatment in leukemia (Figure 3a) (Martens et al. 2010). As another example, *rs2759386* is associated with isoform ratios of the distant gene *FYB* (Figure 3c). Interestingly this effect is mediated through a *cis*-association with a known splicing factor, *QKI*, and the distribution of p-values for *rs2759386* for *trans*-splicing effects across the genome is skewed toward smaller values, suggesting this variant may have effects on splicing of more genes than detectable at global significance levels (Figure S22).

To better understand the causal relationships of *trans*-associated loci (Schadt et al. 2005), we estimate maximum likelihood Bayesian network structure for each *trans* SNP along with its proximal and distal associated genes (Figure 3b, Supplementary Materials). The results indicate that the expression level of nearby genes mediate the *trans* effect 85% of the time, though there is evidence of some residual association in most cases. The remaining 15% of the time, there is little to no explanatory power from expression of nearby genes, and further, in three cases, we observe a paradoxical relationship with the SNP having opposite effects on expression levels of different targets not consistent with correlation *among* the targets (Figure S23). These seemingly paradoxical effects and evidence of incomplete mediation may reflect temporal dynamics, feedback, and non-transcriptional factors such as protein functionality. Overall, these results support a general pattern of modular *trans*-effects mediated by regulatory roles of nearby genes, but a complex architecture for the human regulatory network.

Limitations on deleterious impact from regulatory variation.

Assessing the distribution of eQTLs according to the properties of affected genes, we find evidence supporting mechanisms that limit the deleterious impact of regulatory genetic variation on the cell. First, we observe an inverse relationship between allele frequency and regulatory impact of each SNP, using a subsampling approach to avoid bias in estimates of effect size (Methods). Specifically, *cis*-eQTL effect sizes are significantly smaller for polymorphisms with higher minor allele frequencies (Figure 4a, p < 1e-7), supported by a similar inverse relationship observed between MAF and allelic imbalance among heterozygotes for these polymorphisms (p < .02). This suggests that purifying selective pressure, even among common variants, acts against variation with large effects on transcription, supporting patterns previously reported among disease and functionally annotated genetic variants (Bodmer and Bonilla 2008; Zhu et al. 2011).

Further, there is a depletion of *cis*-eQTLs among genes with annotations suggesting critical roles in cellular functioning. Previous evidence supports selective pressure on network hubs (Fraser et al. 2002; Jordan et al. 2004; Gerstein et al. 2012), and here, we directly demonstrate depletion of regulatory variants among several classes of important genes. In particular, highly conserved genes (those shared with distant species including yeast and zebrafish) and hubs in human protein-protein interaction networks (Methods) are less likely to be influenced by a detectable *cis*-eQTL. Furthermore, effect sizes among detected *cis*-eQTLs are negatively correlated with the number of known protein-protein interactions for the corresponding genes (p < 1e-35) (Figure 4b). Known transcription factors (TFs) are also depleted for *cis*-eQTLs, and, even within TFs, stronger co-expression with target genes implies both a decreased likelihood of detecting a *cis*-eQTL for the TF itself (p < 1e-2) and reduced effect sizes among the eQTLs that we do observe (p < 1e-100) (Figure 4c, S26). This result provides some explanation for the scarcity of *trans*-eQTLs observed in this and other studies (Fehrmann et al. 2011; Fairfax et al. 2012) – in exactly the cases where a *cis*-acting variant affecting one gene could indirectly drive changes in expression of other genes downstream in the regulatory network, there is a depletion of *cis*-eQTLs. These results together suggest that genes whose expression levels have the potential for broad impact on the cell are subject to tighter control on their own expression. Potential limiting mechanisms include selective pressure constraining the frequency of deleterious regulatory variants, compensatory buffering mechanisms, and auto-regulatory feedback.

Genomic properties and prediction of regulatory variants.

The large collection of QTLs from this study affords the opportunity to generalize beyond specific variants identified to their genomic properties, in order to better understand regulatory mechanisms and provide a basis from which to evaluate the regulatory potential of untested variants. First, by combining the catalog of expression and splicing QTLs with detailed annotations from the ENCODE project and others, we explore candidate mechanisms through which genetic variation may perturb regulatory elements and finally expression (Gaffney et al. 2012; Schaub et al. 2012; The ENCODE Project Consortium 2012). Beyond the expected enrichment of both sQTLs and eQTLs near the TSS (Stranger et al. 2012; Veyrieras et al. 2012), sQTLs are concentrated among exonic and intronic loci, with less bias toward the 5' end of the gene than observed for eQTLs (Figure 5a). Further, splice site, essential splice site, and stop gained functional annotations are particularly enriched for sQTLs, beyond the effects of position (Figure 5b, S27) relative to the gene (Methods).

After controlling for SNP proximity and intragenic location, we observe strong enrichment of both eQTLs and sQTLs for many regulatory annotations derived from the ENCODE project, including TF binding sites from ChIP-seq, RNA binding elements from RIP-seq and tiling arrays, and regions of open chromatin identified by DNase I hypersensitivity assays (Methods, Figure 5b, Data S2). While enrichment statistics are not conclusive regarding causality, these observations suggest that variants affecting splicing may act not only through direct perturbation of splice sites and coding loci as has been previously described (Pickrell et al. 2010), but also through TF binding and mechanisms shared with regulation of expression. This could arise from transcription factors playing a role in the cellular pathways that regulate expression of particular isoforms under different conditions, including direct influence on mechanisms such as co-transcriptional splicing. The enrichment of most regulatory elements is strongest among variants close to the TSS and declines in significance with distance for both eQTLs and sQTLs (Figure S27, Data S2), but a small number of transcription factors are enriched for eQTLs at distant locations beyond 100kb, including *MEF2A* (log odds multiplier 0.25). These findings support the hypothesis that a large fraction of regulatory variants affect expression through alteration of transcription factor (TF) binding and chromatin accessibility (Degner et al. 2012), improving the resolution of previous findings and extending these observations to variants associated with gene splicing.

Additionally, we utilize these patterns to develop a predictive model of regulatory variation. With advances in sequencing technology, population and disease studies increasingly identify genetic variants not previously evaluated. Many genetic studies will not include high-resolution expression data or include enough instances of a given allele to accurately evaluate impact on the transcriptome. Thus, it would be valuable to *predict* the impact of non-coding and intergenic variants without requiring direct association testing (Lee et al. 2009; Cooper and Shendure 2011; Gaffney et al. 2012). Our observed associations provide a large set of eQTLs and sQTLs to support the *training* of predictive models to detect regulatory variation. Our approach unifies the full set of informative annotations analyzed for enrichment into a single framework, the Latent Regulatory Variant Model (LRVM, software available at http://dags.stanford.edu/dgn/). LRVM models the likelihood of observing a QTL association based on diverse genomic annotations, using a Bayesian latent variable approach to account for the confounding influences of minor allele frequency and linkage disequilibrium on eQTL association (Methods, Supplementary Materials). In particular, LRVM first models the latent regulatory potential of each individual SNP based on genomic annotations, and separately, the likelihood of observing a QTL association given MAF and LD with surrounding SNPs.

Here, we trained two instances of LRVM, using the described framework but training the parameters separately to predict effects on total expression and, in the second instance, gene splicing. In both cases, using held out genes and SNPs not used in training, we find that LRVM stratifies candidate regulatory SNPs more accurately than any single genomic annotation and more accurately than comparable methods that ignore the effects of LD and MAF (Figure 5c, S28, S29). The SNPs identified by LRVM as having high likelihood of regulatory impact are also more likely to be associated with downstream allelic imbalance (p < 1e-12, Figure 5e), with this stringent test demonstrating that variants identified by LRVM are likely to have true *cis*-regulatory impact. To demonstrate the application of LRVM to interpretation of disease studies, we also predicted the regulatory impact of all known GWAS variants not used in training the model, and interestingly, observe that these variants have an increased probability of regulatory impact (Figure 5d, S30), indicating that many disease SNPs have genomic properties consistent with regulatory function. For example, the top-scoring disease SNP is *rs2932538*, a known cardiovascular risk variant (Ehret et al. 2011) that lies near the TSS of *MOV10* and is annotated with multiple TF binding and DNase hypersensitivity sites, strongly supporting its regulatory potential.

Discussion

Sequencing RNA from primary tissue of nearly one thousand individuals, we identified regulatory variation underlying diverse expression phenotypes of over ten thousand genes across the genome. These results emphasize the pervasive impact of proximal genetic variation on gene expression, and specifically demonstrate a much larger role for genetic variation in gene splicing than previously observed through smaller RNA-sequencing studies (Montgomery et al. 2010; Pickrell et al. 2010) or specialized assays (Kwan et al. 2008). We also identified a number of *distal* regulatory relationships, including a number of distant QTLs affecting isoform ratio within each chromosome (which may still act in *cis*), but proportionally far fewer sQTLs *between* chromosomes. Distant intra-chromosomal and *trans*-eQTLs both include many variants individually acting on groups of multiple genes, supporting observations of modularity in several organisms (Lercher et al. 2002; Oliver and Misteli 2005). We demonstrated that membership of genes in co-regulated modules is predicted, not only by linear proximity, but also by proximity due to three-dimensional chromosomal configuration, extending beyond specific examples of spatial proximity affecting gene regulation (Spilianakis and Flavell 2004; Lieberman-Aiden et al. 2009; Cheung et al. 2010) to suggest a broad, genome-wide pattern.

Furthermore, by analyzing the distribution of eQTLs across the genome, we have detected a depletion of regulatory variants among genes with increased potential for deleterious impact on the cell, including highly conserved genes and genes central to cellular networks. This analysis complements studies identifying evidence of purifying selection in both functionally annotated regulatory and protein-coding elements (Fraser et al. 2002; Jordan et al. 2004; Bodmer and Bonilla 2008; Zhu et al. 2011; Gerstein et al. 2012), directly demonstrating a pattern of reduced impact from regulatory variation for important genes and common variants. Finally, we combined our large set of eQTL with diverse genomic annotations, including location, functional annotations and regulatory elements. We presented a Bayesian model, LRVM, capable of *predicting* regulatory impact from the properties of each SNP, combining all available genomic annotations into a unified model. LRVM improves our ability to predict the effects of genetic variation on both expression and splicing, and provides a framework to automatically identify SNPs likely to have regulatory impact even for variants not previously observed, which could contribute to the interpretation of rare variants and personal genomes.

Overall, the extensive set of eQTLs identified here has provided a more complete characterization of the landscape of human regulatory variation than previously possible. As we seek to describe the impact of both common and rare genetic variation, the combined understanding gained from ongoing genomic annotation efforts, eQTL studies, and statistical methods will contribute to our interpretation of the genetics of gene expression and potential mechanisms of effect on higher-level traits.

Methods

Sample collection. The recruitment procedure of the Depression Genes and Networks study (National Institute of Mental Health grant 5RC2MH089916) was designed to collect samples of individuals with and without major depressive disorder, ages 21 to 60, from a survey research panel that was broadly representative of the United States population (Supplementary Materials). Whole blood was collected in two PAXGene tubes (for RNA) and two acid-citrate-dextrose tubes (for DNA).

Genotyping. DNA was extracted and genotyped on the Illumina HumanOmni1-Quad BeadChip. Quality control was performed to identify samples with elevated heterozygosity, unexpected ancestry or pairwise IBD, and potential mislabeling (Supplementary Materials, Figure S1).

RNA-sequencing and quality control. RNA was extracted from thawed PAXGene tubes and the GLOBINclear^{TM} Kit (Invitrogen) was applied. Sequencing libraries prepared (without fragment size selection) according to the Illumina TruSeq protocol. Oligonucleotide barcodes were attached to all fragments of each library and three libraries at a time were pooled and sequenced in one lane in an Illumina HiSeq 2000

(50bp single-ended reads). FASTQ (Cock et al. 2010), Picard (http://picard.sourceforge.net/), and in-house metrics were used to evaluate data quality (Supplementary Materials, Figures S2-S4).

Expression quantification. RNA-seq reads were mapped to the NCBI v37 H. sapiens reference genome using TopHat (Trapnell et al. 2009). HTSeq was used to quantify gene expression (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html), BEDTools (Quinlan and Hall 2010) was used to quantify exon expression, and Cufflinks (Roberts et al. 2011) was used to quantify isoform expression. Only uniquely aligned reads were used for gene and exon quantification. We consider a gene expressed if it has at least 10 reads in 100 individuals. *Isoform ratio*, representing the *fraction* of a gene's expression arising from a particular transcript, was computed for each transcript from Cufflinks output. In particular, given a gene with *k* transcripts, let e_{ji} represent the expression level of transcript *i* in individual *j.* The isoform ratio for the i^{th} transcript of this gene in individual *j* is given by $\frac{e_{ji}}{\sum_{v=1}^{k} e_{jv}}$,. Allele-specific expression calls were made using a binomial model after filtering for potential mapping bias (Supplementary Materials).

Correcting for potential confounders in RNA-seq data. We used the Hidden Covariates with Prior (HCP) method (Mostafavi et al. 2013) to correct for technical and biological factors including blood cell-type frequencies and the time of the blood draw (see Table S1). We computationally inferred cell-type frequencies using an existing compendium of blood cell-type-specific signatures (Supplementary Materials). HCP parameters were tuned separately for *cis*- and *trans*-eQTL analysis (Supplementary Materials, Figure S7, S8).

Expression QTL mapping. Association testing for *cis*, splicing, and *trans*-eQTLs was performed using Spearman's rank correlation, only including autosomal SNPs with MAF >= 0.025. For *cis*-eQTLs and sQTLs, testing was limited to SNPs within 1Mb of the transcription start site. We used Bonferroni correction to account for the number of SNPs tested per gene (Figure S9), and subsequently identified eQTLs using gene level significance at FDR 0.05. We identified intra-chromosomal eQTLs using stepwise regression (on ranked data for consistency with Spearman's rank correlation). For aseQTL associations, we used a Wilcoxon rank sum statistic indicating whether imbalance of the allelic ratio at the coding locus is significantly higher for individuals heterozygous at the candidate regulatory locus compared to homozygous individuals (all tested individuals are heterozygous at the coding locus). We defined allelic imbalance as $|\frac{r}{r+a} - 0.5|$ where *r* and *a* are the number of reads mapped to the reference and alternative alleles, respectively. We require 10 individuals heterozygous at *both* the coding and candidate regulatory loci.

eQTL and ASE effect size. We estimated eQTL and ASE effect sizes by computing fold change, using a subsampling approach to avoid biases from allele frequency, matching across all tests the number of individuals with each allele analyzed and, in the case of ASE, read depth (Supplementary Materials).

Data sources for analysis of eQTL distribution. We constructed a combined protein-protein interaction network, by taking the union of interactions reported in (1) BioGRID (Breitkreutz et al. 2003) (both small scale and high-throughput), (2) MINT (Chatr-aryamontri et al. 2007), (3) HPRD (Goel et al. 2012), and (4) IntAct (Aranda et al. 2010) (all data obtained from the GeneMANIA (Warde-Farley et al. 2010) data repository (download on 01/4/2012)). Transcription factor (TF) target information was downloaded from the ChEA database (Lachmann et al. 2010). Conserved genes were obtained for each species from BioMart (Haider et al. 2009). All analyses were restricted to protein coding genes. We obtained trait and disease associated SNPs (GWAS hits) (Hindorff et al. 2009) (downloaded from NHGRI on 01/26/2011), and only considered SNPs with an association of p <= 1e-7.

***Trans*-eQTL detection and filtering**. To account for spurious *trans*-associations that stem from regions of sequence similarity and potential mapping errors, we devised a series of filters and applied them to the candidate *trans*-eQTLs. These filters exclude associations that involve pseudogenes (Karro et al. 2007), associations within paralog families (Vilella et al. 2009), associations between regions of high sequence similarity based on read mapping simulation, and associations that fail a “smoothness” test which evaluates the association signal across the expressed exons of a gene (Supplementary Materials, Figure S20).

Analysis of enrichment of QTLs in genomic annotations. We collected genomic annotations from RegulomeDB (Boyle et al. 2012), collected RNA-binding data from ENCODE (The ENCODE Project Consortium 2012) (both the RIP-seq and tiling array data were used), and positional annotations from the Ensembl Variant Effect Predictor (McLaren et al. 2010). To accurately estimate the effects of each genomic annotation on the likelihood of a SNP having regulatory impact, we first account for the location of each SNP. We categorized SNPs within the gene boundaries as exonic, intronic, or UTR, with separate categorization for the first exon and intron. Then, SNPs not within the corresponding gene boundaries are categorized into upstream and downstream regions of size 1kb (for loci within 10kb of the TSS), and 10kb (regions beyond 10kb). For each positional category, we compute the odds multiplier on observing an association using logistic regression. Fixing these parameters, we then compute the adjusted odds for each SNP based on position and find the *conditional* odds multiplier for each genomic annotation beyond the effects of location. We consider regions within 20kb, 20kb-100kb, and over 100kb from the TSS separately for enrichment analysis. To ensure that the enrichment signal is not mainly driven by annotations from a small number of genes or unusual genomic region, we repeated the analysis excluding each chromosome independently, and reported the minimum enrichment among these twenty-two estimates for each genomic feature. (See Data S2 for the complete enrichment results).

Regulatory variant predictive model (LRVM). We defined a Latent Regulatory Variant Model (LRVM) (Figure S28a), a Bayesian network modeling the probability that each SNP will be associated with an expression trait based on genomic annotations. As we describe in details below, in order to account for associations that arise from linkage disequilibrium (LD) with a nearby eQTL, while still maintaining the ability to capture *multiple* independent eQTLs impacting the same gene, we model all SNPs near the same gene jointly. In addition, we also account for the effects of allele frequency (which can affect statistical power to detect a an association even for causal SNPs). This framework is used for prediction of both eQTL and sQTL associations, but model parameters are trained separately to reflect differences in their genomic properties.

For each gene, we identify the set of candidate eQTL SNPs ($s_1, \ldots, s_k$) in some chosen window near the transcription start site (TSS) of the gene (in the analysis of LRVM we focus on SNPs within 20kb of the TSS). For each of these SNPs, we define a binary variable a_i representing whether SNP i is associated with the gene, along with a separate *latent* binary variable d_i representing whether SNP i itself has strong regulatory potential. Using the location features and genomic annotations available for SNP i as a feature vector F_i (see previous section for location and genomic annotations used in this study), we define a logistic function, parameterized by a shared set of weights w, to predict d_i from F_i. The association variables a_i are also predicted using a logistic function, where the input to each a_i is the full vector of latent variables ($d_1 ... d_k$) for all nearby SNPs, each weighted by their MAF and LD with *i.* This model is summarized as follows:

$$p(d_i|F_i, \boldsymbol{w}) = \frac{1}{1+e^{-F_0-F_i w}}\ , \ \ p(w) \sim N(0,1) \qquad (4)$$

$$p(a_i|\boldsymbol{d}) = \frac{1}{1+e^{-\gamma_0-\alpha m_i d_i+\beta m_i(\sum_{k\neq i}(l_{k,i} d_k))}} \qquad (5),$$

with $l_{k,i}$ indicating the LD between i and k^{th} SNP, m_i indicating the MAF of i^{th} SNP, parameters β and α modulating the effects of LD and MAF, and parameters γ_0 and F_0 indicating the prior odds. From a training set comprised of features F_i and observed a_i from eQTL association results, we learn the parameters w, β and α using Hard Expectation Maximization (Dempster and Laird 1977). In this method, we iterate between finding the most likely assignment to latent variables d using a current estimate of w, β and α, and re-estimating w, β and α using the current assignment to d. We can then test the accuracy of our model by evaluating its ability to predict associations on a held out set of genes and SNPs (Figure S28b, S29, S31). Separate models (different estimates of w, β and α) are learned for eQTLs and sQTLs. See Supplementary Materials, and Figures S28b, S29, S31, for description of evaluation and comparisons of LRVM with other models.

Data access

Genotype, raw RNA-seq, quantified expression, and covariate data are available by application through the NIMH Center for Collaborative Genomic Studies on Mental Disorders. Instructions for requesting access to data can be found at https:www.nimhgenetics.org/access_data_biomaterial.php, and inquiries should reference the “Depression Genes and Networks study (D. Levinson, PI)”.

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Author contributions

AB designed and carried out the analysis, with input from DK, DFL, SBM, SM, and XZ. SM contributed to development and implementation of statistical analysis. DFL and DK designed the Depression Genes and Networks study, with contributions from AEU. XZ developed the RNA-seq quantification and QC pipeline and contributed to bioinformatic analysis. JBP and MMW performed clinical interviews for identifying appropriate subjects. CM and CDH carried out the RNA sequencing. KBB performed initial RNA sample preparation. JS performed the genotype QC. RM carried out the genotyping.

Disclosure declaration

CM is an employee of Illumina, Inc. CDH was employed by Illumina, Inc. for a portion of the study and by Personalis for a portion. RM is employed by Centrillion Biosciences, Inc.

Figure legends

Figure 1: *Cis*-regulatory variation and allelic effects

a) Schematic illustration of aseQTL. Heterozygosity at a regulatory locus is linked to allelic imbalance detected from RNA-seq reads over a *separate* heterozygous coding SNP (a second, separate locus) in the corresponding gene. Conversely, individuals who are homozygous at the regulatory SNP will show balanced allelic expression at the coding SNP (still estimated among individuals who are heterozygous at the coding SNP).

b) Example aseQTL, the most significant association in this analysis. *Rs4950928*, a known asthma risk variant SNP in the 5' UTR of *CHI3L1*, is associated with allelic imbalance in the coding region of *CHI3L1*, with heterozygous individuals showing significantly increased allelic imbalance compared to individuals homozygous for either the reference or non-reference allele (p < 1e-71, Methods).

c) Distribution of significant ASE by individual. In each individual, we evaluate the fraction of testable heterozygous loci (requiring sufficient read depth and other filters, see Supplementary Materials) with significant ASE (binomial p <= 1e-3). To evaluate the distribution of ASE *not* explained by heterozygosity for a common regulatory variant, we then evaluate the same set of testable loci, but only counting ASE when the individual is not heterozygous for a corresponding eQTL SNP. In this case, we consider SNPs that are significant at p <= 1e-3 for the corresponding eQTL gene.

Figure 2: Distant and modular intra-chromosomal regulation

a) Q-Q plot of aseQTL p-values for intra-chromosomal eQTLs of varying distances. For eQTLs implicating SNPs beyond each distance threshold from the corresponding TSS

(0kb, 20kb, 100kb, 300Kb, 500kb), we computed aseQTL association tests between the eQTL SNP and allelic ratios at all exonic loci available for the corresponding gene, taking the best association identified from these. The expected p-value distribution and 95% CIs were computed empirically from repeated *random* draws of SNPs similarly tested against exonic loci within each eQTL gene. We observe that distant eQTLs show more ASE than expected by chance, though the enrichment declines with distance.

b) Schematic of a genomic region on chromosome 16 containing co-regulated genes, along with nearby genes and SNPs having impact on each gene. *Rs11644386* affects a dis-continuous group of genes, with the farthest association (*CYLD*) being over 400kb away, and does not have significant associations with two intermediate genes *SNX20* and *NOD2*. Another SNP, *rs8047222,* is associated with expression of *NKD1* and a nearby gene *NOD2*, but has no influence on the more distant genes *BRD7* and *ADCY7*.

Figure 3: *Trans* regulatory variation and mediation through proximal genes

a) Example sub-network of significant associations centered on expression of *IKZF1*. The SNP *rs10251980* is associated with expression of the nearby gene *IKZF1* (p < 1e-8), along with eight distant genes (p < 1e-12). *IZKF1* is also co-expressed with six of the eight genes (p < 0.05).

b) Prevalence of candidate regulatory network structures for *trans*-eQTLs including the SNP, the corresponding distant genes, and any genes proximal to the SNP that are also associated with its genotype. For each *trans*-eQTL gene, we analyzed its relationship with the most strongly associated SNP, along with all genes within 1Mb of that SNP. Network structures best fitting each set were identified using likelihood ratio tests (Methods, Supplementary Materials).

c) Association between *rs2759386* and isoform ratio of *FYB*, potentially mediated through expression of splicing factor *QKI*, which is proximal to the SNP. *Rs2759386* is associated with total expression levels of *QKI*, and both this SNP and *QKI* are associated with isoform ratio of *FYB* (p < 1e-14 and p < 1e-16, respectively).

Figure 4: Distribution of *cis*-regulatory variation and selective pressure

a) Effect size of *cis*-eQTLs compared to minor allele frequency of the most significant SNP per eQTL gene (computed using subsampling, Methods). We find a strong inverse relationship (Spearman's r = -.13, p < 1e-7). If we normalize by the observed variance of each gene, the observed relationship becomes stronger (p < 1e-39).

b) A depletion of *cis*-eQTLs is evident (p < .05) among genes with many protein-protein interactions (PPI) and, additionally, a strong negative correlation between the number of interactions and eQTL effect size (p < 1e-35). Protein coding genes were put into quantile buckets according to the number of known PPI relationships (Methods). The fraction of genes in each bucket having a significant *cis*-eQTL was computed, along with the average effect size for the observed eQTLs. Fewer eQTLs are observed among genes with the most interactions (hub genes). Genes in the bottom 20% may be moderately depleted as well, though confidence intervals (95%) are overlapping with the intermediate deciles.

c) The fraction of genes with a significant *cis*-eQTL and average eQTL effect size are shown according to an estimate of the genes' regulatory impact. Known regulatory genes were put into quantiles according to the strength of correlation observed between their expression profile and the expression of all non-regulatory. Non-regulatory genes are shown in the left-most bar for comparison. Strong regulatory genes show significant depletion of eQTLs (p < 1e-2) compared to non-regulators and weak regulatory genes and, similarly, reduced eQTL effect sizes (p < 1e-100).

Figure 5: Genomic properties of regulatory variation and prediction of eQTLs

a) Enrichment of proximal eQTLs and sQTLs is shown as a function of distance to the TSS. Enrichment is computed here as the log odds multiplier on likelihood of association (Methods). In the zoomed, intrageneic view, enrichment (log odds multiplier) of proximal eQTLs and sQTLs is shown within gene boundaries for UTR, intronic, and exonic loci. We aggregate SNPs within all exons except the first (the closest to TSS) together, and likewise for introns.

b) Enrichment of *cis*-eQTLs and sQTLs for functional and genomic annotations, controlling for distance. In each case, (log) odds multipliers were computed for each category after conditioning on SNP location (Methods) shown in (a) and (b). ChIP-seq and DNase I annotation enrichments are shown here for SNPs falling within 20kb upstream of TSS; for full enrichment statistics see Data S2.

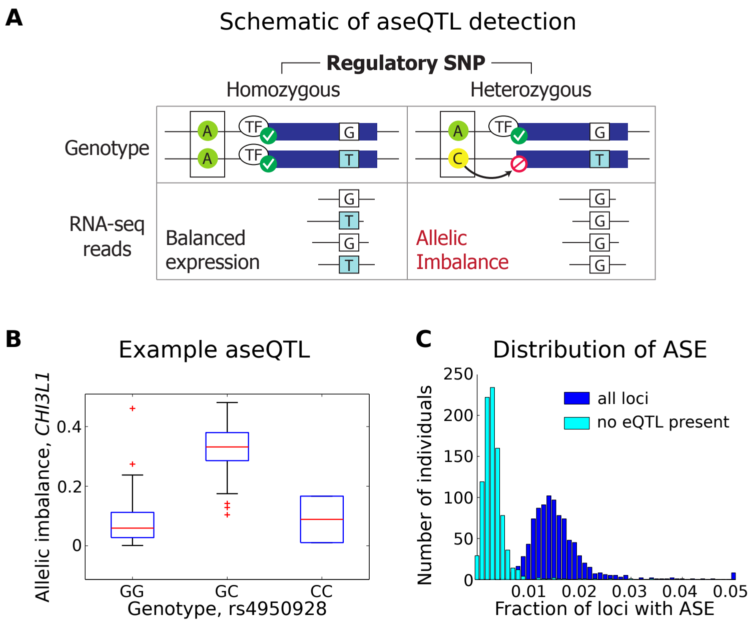
c) Enrichment of *cis*-eQTLs stratified by LRVM score (restricting to genes and SNPs excluded from training LRVM). Each SNP-gene pair was scored by LRVM for likelihood of association, and twenty quantiles were computed for the resulting scores. Finally, enrichment was computed for each quantile, using log odds estimation after correcting for position (Methods).

d) Predicted regulatory impact of trait-associated (GWAS) SNPs according to LRVM, for 263 unique disease variants not available during LRVM training. We compute the score of each SNP for each of its proximal genes. Known trait-associated SNPs score more highly that expected at random ($p < 1e-9$), indicating enrichment for properties that match those of observed regulatory variants.

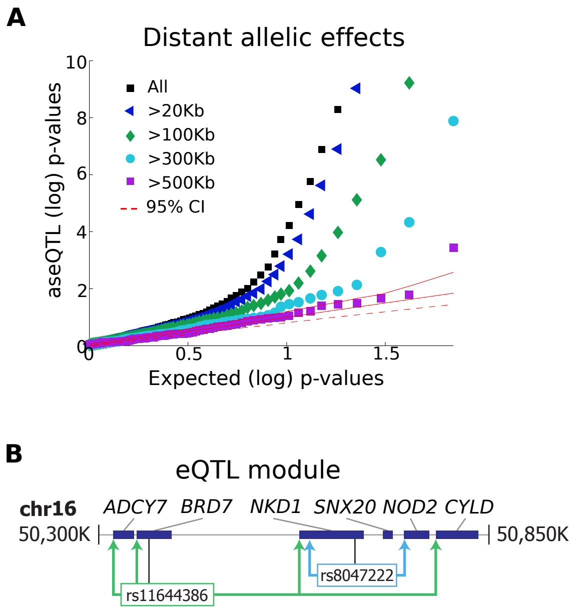
e) LRVM scores are predictive of allelic effects, indicative of *cis*-regulatory impact. We correlate allelic imbalance (Methods) observed among heterozygous individuals for each SNP with the score assigned by each predictive model to the corresponding SNP. Significance is estimated using the Wilcoxon rank sum test. Again, analysis is restricted to SNPs not used to train LRVM.

Figures

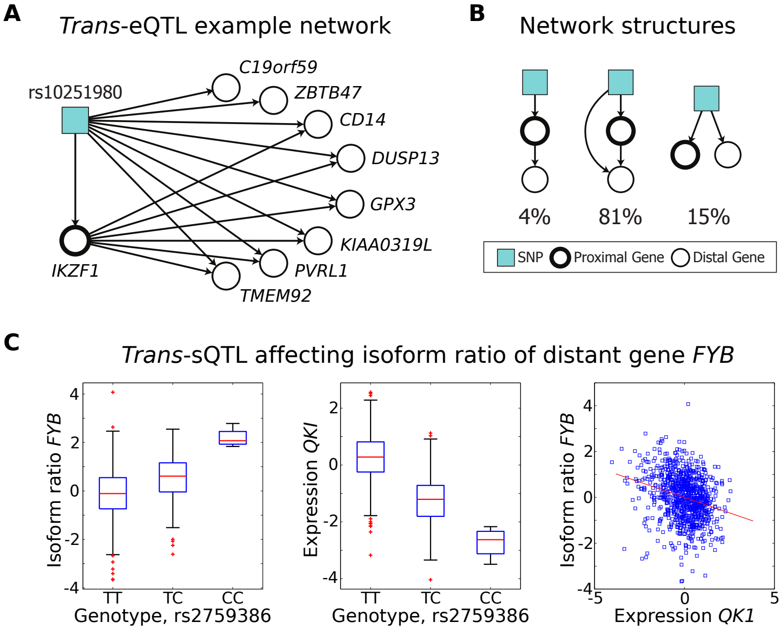
1.


A
Schematic of aseQTL detection
Regulatory SNP
Homozygous
Heterozygous
Genotype
A
A
TF
TF
G
T
A
C
TF
G
T
RNA-seq reads
Balanced expression
G
T
G
T
Allelic Imbalance
G
G
G
G
B
Example aseQTL
Allelic imbalance, CHI3L1
0.4
0.2
0
GG
GC
CC
Genotype, rs4950928
C
Distribution of ASE
250
200
150
100
50
0
Number of individuals
all loci
no eQTL present
0.01
0.02
0.03
0.04
0.05
Fraction of loci with ASE

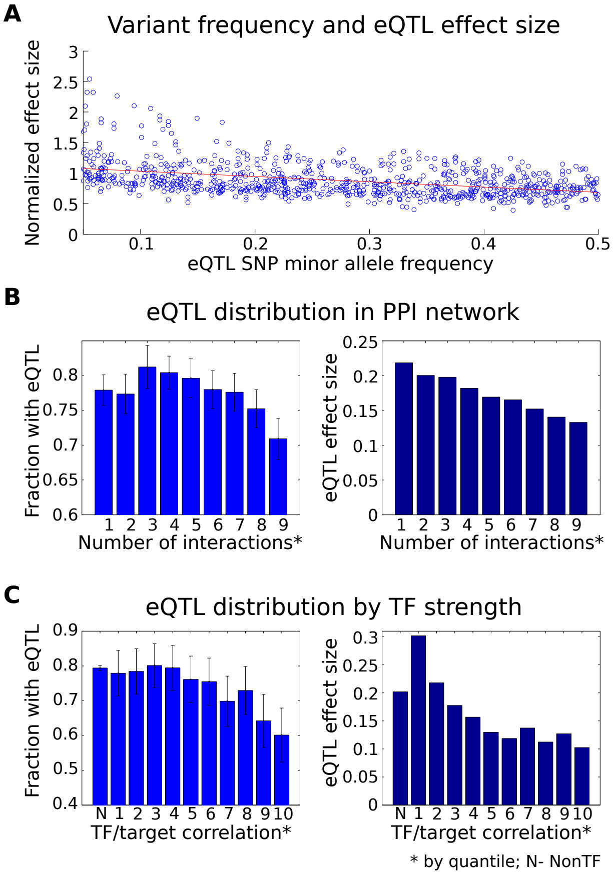
2.


A
Distant allelic effects
10
8
6
4
2
0
aseQTL (log) p-values
■ All
◀ >20Kb
◆ >100Kb
● >300Kb
■ >500Kb
-- 95% CI
0
0.5
1
1.5
Expected (log) p-values
B
eQTL module
chr16
50,300K
ADCY7
BRD7
NKD1
SNX20
NOD2
CYLD
50,850K
rs8047222
rs11644386

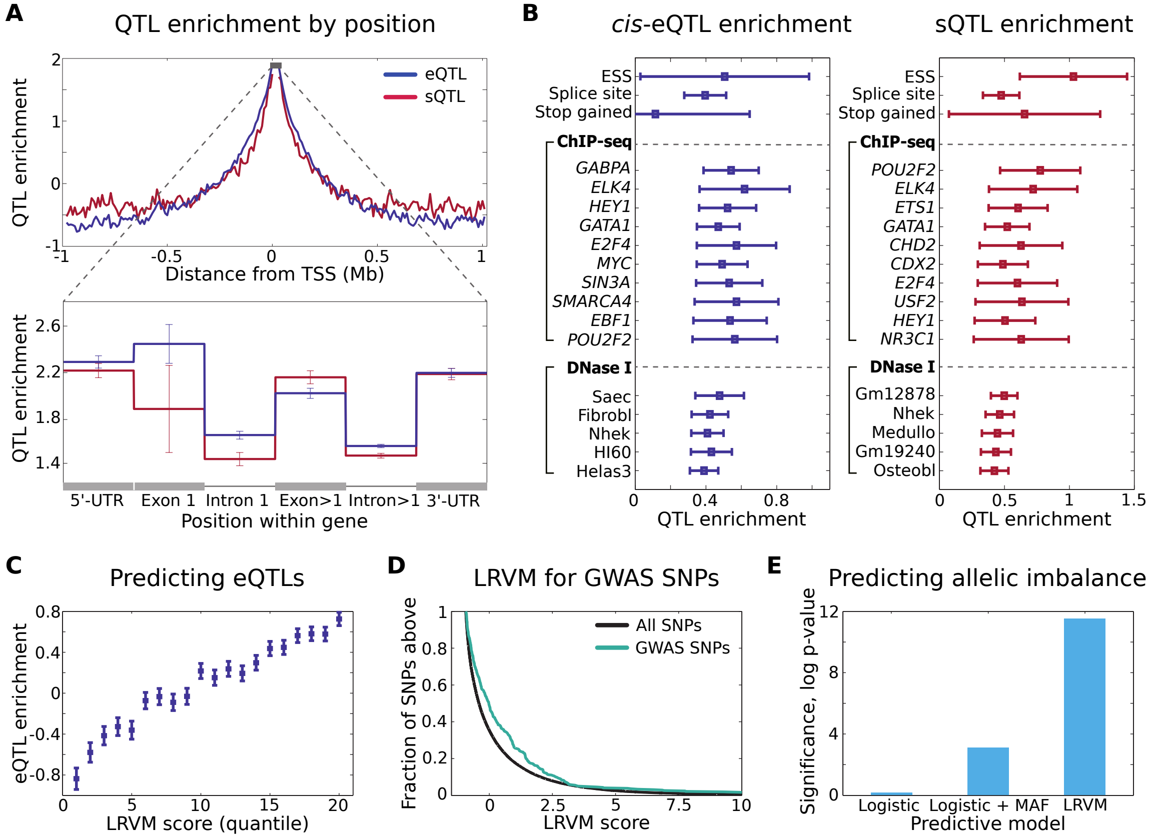
3.


A
Trans-eQTL example network
rs10251980
C19orf59
ZBTB47
CD14
DUSP13
GPX3
KIAA0319L
PVRL1
TMEM92
IKZF1
B
Network structures
4%
81%
15%
SNP
Proximal Gene
Distal Gene
C
Trans-sQTL affecting isoform ratio of distant gene FYB
Isoform ratio FYB
4
2
0
-2
-4
TT
TC
CC
Genotype, rs2759386
Expression QKI
2
0
-2
-4
TT
TC
CC
Genotype, rs2759386
Isoform ratio FYB
4
2
0
-2
-4
-5
0
5
Expression QK1

4.


A
Variant frequency and eQTL effect size
Normalized effect size
3
2.5
2
1.5
1
0.5
0
0.1
0.2
0.3
0.4
0.5
eQTL SNP minor allele frequency
B
eQTL distribution in PPI network
Fraction with eQTL
0.8
0.75
0.7
0.65
0.6
1 2 3 4 5 6 7 8 9
Number of interactions*
eQTL effect size
0.25
0.2
0.15
0.1
0.05
0
1 2 3 4 5 6 7 8 9
Number of interactions*
C
eQTL distribution by TF strength
Fraction with eQTL
0.9
0.8
0.7
0.6
0.5
0.4
N 1 2 3 4 5 6 7 8 9 10
TF/target correlation*
eQTL effect size
0.3
0.25
0.2
0.15
0.1
0.05
0
N 1 2 3 4 5 6 7 8 9 10
TF/target correlation*
* by quantile; N- NonTF

5.


A
QTL enrichment by position
eQTL
sQTL
QTL enrichment
2
1
0
-1
-1
-0.5
0
0.5
1
Distance from TSS (Mb)
2.6
2.2
1.8
1.4
5'-UTR
Exon 1
Intron 1
Exon>1
Intron>1
3'-UTR
Position within gene
B
cis-eQTL enrichment
sQTL enrichment
ESS
Splice site
Stop gained
ChIP-seq
GABPA
ELK4
HEY1
GATA1
E2F4
MYC
SIN3A
SMARCA4
EBF1
POU2F2
DNase I
Saec
Fibrobl
Nhek
HI60
Helas3
0
0.4
0.8
QTL enrichment
ESS
Splice site
Stop gained
ChIP-seq
POU2F2
ELK4
ETS1
GATA1
CHD2
CDX2
E2F4
USF2
HEY1
NR3C1
DNase I
Gm12878
Nhek
Medullo
Gm19240
Osteobl
0
0.5
1
1.5
QTL enrichment
C
Predicting eQTLs
eQTL enrichment
0.8
0.4
0
-0.4
-0.8
0
5
10
15
20
LRVM score (quantile)
D
LRVM for GWAS SNPs
All SNPs
GWAS SNPs
Fraction of SNPs above
1
0.8
0.6
0.4
0.2
0
0
2.5
5
7.5
10
LRVM score
E
Predicting allelic imbalance
Significance, log p-value
12
8
4
0
Logistic
Logistic + MAF
LRVM
Predictive model

Tables

Table 1: Expression quantitative trait loci detected.

	Within 1Mb	**Intra-chromosomal, > 1Mb**	**Inter-chromosomal**	**Transcripts tested**
Total gene expression	10,914	269	138	13,857
Isoform ratio	2,851 (1,370 genes)	80 (56 genes)	5 transcripts (3 genes)	11,227 (4,100 genes)
Allelic ratio	936	N/A	N/A	22,708 (coding loci)

Number of QTLs associated with each expression trait for analyses encompassing varying scopes, all reported at FDR 0.05. Many SNPs affect more than one expression trait. For instance, 28% of cis-sQTL SNPs overlap with a cis-eQTL (p-value within two orders of magnitude of the best eQTL SNP), indicating significant overlap but mostly distinct regulatory effects. Full list of detected eQTLs provided in Data S1.

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