Expression Quantitative Trait Loci Are Highly Sensitive to Cellular Differentiation State

Alice Gerrits1*, Yang Li2*, Bruno M. Tesson2*, Leonid V. Bystrykh1, Ellen Weersing1, Albertina Ausema1, Bert Dontje1, Xusheng Wang3,4, Rainer Breitling2, Ritsert C. Jansen2, Gerald de Haan1*

1Department of Cell Biology, Section Stem Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, 2Groningen Bioinformatics Centre, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands, 3Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, 4Institute of Bioinformatics, Zhejiang University, Hangzhou, China

Abstract

Genetical genomics is a strategy for mapping gene expression variation to expression quantitative trait loci (eQTLs). We performed a genetical genomics experiment in four functionally distinct but developmentally closely related hematopoietic cell populations isolated from the BXD panel of recombinant inbred mouse strains. This analysis allowed us to analyze eQTL robustness/sensitivity across different cellular differentiation states. Although we identified a large number (365) of “static” eQTLs that were consistently active in all four cell types, we found a much larger number (1,283) of “dynamic” eQTLs showing cell-type-dependence. Of these, 140, 45, 531, and 295 were preferentially active in stem, progenitor, erythroid, and myeloid cells, respectively. A detailed investigation of those dynamic eQTLs showed that in many cases the eQTL specificity was associated with expression changes in the target gene. We found no evidence for target genes that were regulated by distinct eQTLs in different cell types, suggesting that large-scale changes within functional regulatory networks are uncommon. Our results demonstrate that heritable differences in gene expression are highly sensitive to the developmental stage of the cell population under study. Therefore, future genetical genomics studies should aim at studying multiple well-defined and highly purified cell types in order to construct as comprehensive a picture of the changing functional regulatory relationships as possible.

Introduction

Genetical genomics uses quantitative genetics on a panel of densely genotyped individuals to map genomic loci that modulate gene expression [1]. The quantitative trait loci identified in this manner are referred to as expression quantitative trait loci, or eQTLs [2]. Most genetical genomics studies that have thus far been reported have analyzed single cell types or compared developmentally unrelated and distant cell types [3–8]. Here, we report the first application of genetical genomics to study eQTL dynamics across closely related cell types during cellular development. We show results that discriminate between eQTLs that are consistently active or “static” and those that are cell-type-dependent or “dynamic.”

We used the hematopoietic system as a model to analyze how the genome of a single stem cell is able to generate a large variety of morphologically and functionally distinct differentiated cells. Differentiation of hematopoietic stem cells towards mature, lineage-committed blood cells is associated with profound changes in gene expression patterns. The search for differentially expressed genes, most notably for those transcripts exclusively present in stem cells and not in their more differentiated offspring, has been successful and has provided valuable insight into the molecular nature of stem cell self-renewal [9–12]. Yet, complementary approaches were needed to elucidate the dynamic regulatory pathways that are underlying the robust differentiation program leading to blood cell production.

We describe a genetic analysis of variation in gene expression across four functionally distinct, but developmentally related hematopoietic cell populations. Our data reveal complex cell-stage specific patterns of heritable variation in transcript abundance, demonstrating the plasticity of gene regulation during hematopoietic cell differentiation.

Results

Genetic Regulation of Gene Expression

We evaluated genome-wide RNA transcript expression levels in purified Lin″c-Kit+ multi-lineage cells, committed Lin−Sca-1−c-Kit+ progenitor cells, erythroid TER-119+ cells, and
Author Summary

Blood cell development from multipotent hematopoietic stem cells to specialized blood cells is accompanied by drastic changes in gene expression for which the triggers remain mostly unknown. Genetical genomics is an approach linking natural genetic variation to gene expression variation, thereby allowing the identification of genomic loci containing gene expression modulators (eQTLs). In this paper, we used a genetical genomics approach to analyze gene expression across four developmentally close blood cell types collected from a large number of genetically different but related mouse strains. We found that, while a significant number of eQTLs (365) had a consistent “static” regulatory effect on gene expression, an even larger number were found to be very sensitive to cell stage. As many as 1,283 eQTLs exhibited a “dynamic” behavior across cell types. By looking more closely at these dynamic eQTLs, we show that the sensitivity of eQTLs to cell stage is largely associated with gene expression changes in target genes. These results stress the importance of studying gene expression variation in well-defined cell populations. Only such studies will be able to reveal the important differences in gene regulation between different cell types.

myeloid Gr-1+ cells, isolated from the bone marrow of ~25 genetically related and fully genotyped BXD – C57BL/6 (B6) X DBA/2 (D2) – recombinant inbred mouse strains [13]. In this study, we exploit the fact that the purified cell populations are closely related, sometimes just a few cell divisions apart on the hematopoietic trajectory. The Lin -Sca-1-c-Kit+ cell population contains all stem cells with long-term repopulating ability, but also includes multipotent progenitors that still have lymphoid potential. Although long-term repopulating stem cells are known to only make up a fraction of the Lin -Sca-1-c-Kit+ population, for simplicity we will refer to this population as stem cells. The Lin -Sca-1-c-Kit+ cell population does not contain stem cells and lymphoid precursors, but does include common progenitors of the myeloid and erythroid lineages [14]. Finally, TER-119+ and Gr-1+ cells are fully committed to the erythroid and myeloid lineages, respectively. Unsupervised clustering of the most varying transcripts demonstrated that each of the four cell populations could easily be recognized based on expression patterns across all four cell types (Figure 1 and Table S1).

We observed strong and biologically significant variation in gene expression during hematopoietic differentiation, independent of mouse strain. However, the genetical genomics strategy, in which we focus on inter-strain gene expression differences, allows for a far more comprehensive understanding of the genetic regulatory links underlying this variation. QTL mapping of gene expression traits allows us to identify eQTLs genomic regions that have a regulatory effect on those expression traits. Two types of eQTLs can be distinguished, i.e., those that map near (less than 10 Mb from) the gene which encodes the transcript (local) and those that map elsewhere in the genome (distant) [13]. Together, local and distant eQTLs constitute a genome-wide overview of the gene regulatory networks that are active in the cell type under study. The strongest eQTLs were found for genes that were expressed only in mouse strains carrying one specific parental allele, suggesting that local regulatory elements are distinct between the two alleles. Cases of such allele-specific expression included H2-Ob and Apobec3. These transcripts were only detectable in strains that carried the B6 allele of the gene (see Figure S1A, S1B). A global view of heritable variation in gene expression indicated that the strongest eQTLs are not associated with the most highly expressed genes, and that for most probes the expression difference between the B6 and D2 alleles is small (see Figure S1C, S1D).

Since the focus of this project is to study the influence of cellular differentiation state on regulatory links, we used ANOVA to distinguish between “static” eQTLs that show consistent genetic effects across the four cell types and “dynamic” eQTLs that are sensitive to cellular state (i.e., eQTLs that have a statistically significant genotype-by-cell-type interaction). We further partitioned dynamic eQTLs into different categories on the basis of their dynamics along the differentiation trajectory.

Cell-Type–Independent Static eQTLs

The first eQTL category comprises genes that have static eQTLs across all four cell types under study. Variation in Lxn expression was shown as a representative example (Figure 2A, left panel). Lxn expression has previously been shown to be higher in B6 stem cells compared to D2 stem cells, and to be negatively correlated with stem cell numbers [16]. In our dataset Lxn showed clear expression dynamics (it was most highly expressed in stem cells), and was indeed more strongly expressed in cells carrying the B6 allele, but the expression differences between cells expressing the B6 or D2 allele remained constant across all cell types.

In total, we identified 365 probes that displayed a static eQTL at threshold $p < 10^{-6}$ (FDR $= 0.02$). Among the 268 locally-regulated probes in this category was H2-D1. The histocompatibility gene H2-D1 is known to be polymorphic between B6 and D2 mice, and would therefore be expected to be in the static eQTL category. The remaining 97 probes mapped to distant eQTLs, i.e., their heritable expression variation was affected by the same distant locus in all four cell types (Table 1).

All probes that belonged to the static eQTL category are graphically depicted in an eQTL dot plot displaying the genomic positions of the eQTLs compared to the genomic positions of the genes by which the variably expressed transcripts were encoded (Figure 2A, right panel). Whereas in this plot local eQTLs appear on the diagonal, distant eQTLs appear elsewhere. In general, as has been reported before in eQTL studies, transcripts that were locally regulated showed strong linkage statistics. Not surprisingly, the statistical association between genotype and variation in transcript abundance for those transcripts that were controlled by distant loci was weaker. These genes are likely to be controlled by multiple loci, each contributing only partially to the phenotype, thereby limiting their detection and validation in the current experimental sample size. A list of all transcripts with significant static eQTLs is provided in Table S2.

Cell-Type–Dependent Dynamic eQTLs

The second eQTL category comprises genes that have dynamic eQTLs across all four cell types under study. In total, we identified 1283 eQTLs ($p < 10^{-6}$, FDR $= 0.021$) that showed different genetic effects in different cell types, indicating that eQTLs are highly sensitive to cellular differentiation state (Table 1). Within this dynamic eQTL category, the first four subcategories are composed of eQTLs that were preferentially active in only one of the four cell types we analyzed (Figures 2B–2E).

For example, Slit2 mapped to a strong eQTL that was active only in stem cells. Slit2 mRNA was only detected in the most primitive hematopoietic cell compartment in those BXD strains that carried the D2 allele at rs13478235, a SNP that mapped 629 kb away from the Slit2 gene (Figure 2B, left panel). Slit2 encodes an excruted chemorepellent molecule that is known to be
expressed in embryonic stem cells [17], to be involved in neurogenesis [18] and angiogenesis [19], and to inhibit leukocyte chemotaxis [20]. We found a total of 140 genes that have eQTLs that are preferentially/selectively active in stem cells (Figure 2B, right panel, largest symbols, Table 1). These 140 genes included well-known candidate stem cell genes such as Angpt1, Ephb2, Ephb4, Foxa3, Fzd6, and Hoxb5. Interestingly, many transcripts with as yet unknown (stem cell) function were transcriptionally affected by stem-cell-specific eQTLs. Candidate novel stem cell genes include Msh5, and Trim47, in addition to a large collection of completely unannotated transcripts.

A total of 45, 53, 1, and 295 eQTLs were found to be preferentially/selectively active in progenitors, erythroid cells, and myeloid cells, respectively (Table 1). Very distinct patterns of cell-type–specific gene regulation emerged when these eQTLs were visualized in genome-wide dot plots (Figures 2C–2E). Using genome-wide \( p \)-value thresholds of \( p < 10^{-6} \), we identified 53 distantly-regulated transcripts in stem cells, 13 in progenitor cells, 400 in erythroid cells, and 132 in myeloid cells. In erythroid and myeloid cells most of these transcripts mapped to relatively few genomic loci; these trans-bands are statistically significant, as assessed by a permutation approach taking expression correlation into account [see Materials and Methods] [21]. Typically, transcripts mapping to a common marker showed a directional bias towards either B6 or D2 expression patterns.

In addition to the relatively simple eQTL dynamics that we have thus far illustrated, more complex eQTL dynamics were also detected using this approach. For example, Rpo1-2 is a transcript that shows a strong local eQTL in the two non-committed lineages included in our study, but shows a much weaker genetic effect in erythroid and myeloid cells (Figure 2F). Whereas in mice carrying the B6 allele of Rpo1-2 the overall expression of the gene decreased substantially during differentiation of progenitor to erythroid cells, in mice carrying the D2 allele expression slightly increased. This observation hints at complex regulatory mechanisms underlying the expression of this gene. Full lists of genes in each dynamic eQTL subcategory described thus far are supplied in Table S2. Additional subcategories and their exact definitions are explained more extensively in the Materials and Methods section, and complete results of all dynamic eQTLs are available in Table S3.

**Detailed Analysis of Static and Dynamic eQTLs**

eQTL dynamics can be caused by transcription factors being switched on/off upon cellular differentiation, or by a transcription factor showing changed specificity due to variations in regulatory input. We found that most (>75%) of the dynamic eQTLs are active in only one of the four cell types under study (Figure 3A). A more detailed analysis revealed that in the majority of cases the genes with a cell-type-specific eQTL were also most highly expressed in that particular cell type (Figure 3B). Next, we
explored whether we could find transcripts that were regulated by distinct eQTLs in different cell types (see Materials and Methods). Such eQTL “swapping” would indicate major changes in transcriptional regulatory networks. We could find no evidence for such cases. However, given our limited population size we have a low power to detect multiple eQTLs, so swapping eQTLs may still exist but remain undetected in our experimental setting.

It has been described that not all local eQTLs in genetical genomics experiments reflect actual expression differences between mouse strains, but rather indicate differential hybridization caused by polymorphisms in the sequences recognized by the probes [22]. For this reason, we divided both the static and dynamic eQTL categories in local and distant eQTLs, and indicated the number of probes that hybridized to sequences that are known to contain polymorphisms (Figure 3C). As expected, the static eQTL category contained a higher number of such potential false local eQTLs. If these false positive eQTLs could be removed, the relative abundance of dynamic eQTLs would be higher, indicating that our study may even conservatively underestimate the level of eQTL dynamics.

Discussion

We found that many eQTLs are highly sensitive to the developmental state of the cell population under study. Even when the purified cells were only separated by a few cell divisions, eQTLs demonstrated a remarkable plasticity. Furthermore, we provide evidence that the cell-stage-sensitivity of eQTLs is often
We did not identify target genes that were regulated by distinct eQTLs in different cell types, suggesting that large-scale changes within transcriptional regulatory networks are not common. The fact that eQTLs appear to be highly cell-type–dependent highlights the importance of using well-characterized purified cell types in eQTL studies. In particular, eQTL studies of physiological or disease processes [23–26] should target the relevant cell type as precisely as possible, i.e. they should use cells or tissues directly involved in the pathophysiological process. This could even mean that several different cell types need to be separately studied, in particular if developmental trajectories are affected [27]. Using unfractionated bone marrow cells, we would have missed many of the diverse and dynamic patterns that we uncovered here, both at the expression level and at the genetic regulatory level. Even so, the four cell populations that we studied are still heterogeneous and further subfractionation of these populations based on different sets of markers would have resulted in even more precise regulatory maps.

Many genetical genomics experiments have used highly heterogeneous samples, in which mRNA from a variety of different cell types was pooled [4,5,28–31]. In such mixed samples it is usually impossible to ensure that the contribution of individual cell types to the mixture is the same across samples. As a result, important parts of the variation in gene expression could arise from different sample compositions. For example, if in whole brain samples a heritable morphological or developmental trait leads to an increased size of some brain regions, this can cause apparent hotspots for transcripts that are specific for those particular regions. Our data provide a valuable tool for studying the exact consequences of sample heterogeneity on eQTL mapping: a further study could simulate a collection of samples made of computed mixtures of different hematopoietic cells in defined proportions. Clearly, cell purification strategies are essential to identify those cell-type–specific eQTLs that would otherwise be “masked” in heterogeneous cell populations. Therefore, future genetical genomics studies should be realized on as many cell types or cellular differentiation states as possible, and ideally even on the scale of individual cells.

All data presented in this paper were deposited in the online database GeneNetwork (http://www.genenetwork.org), an open web resource that contains genotypic, gene expression, and phenotypic data from several genetic reference populations of multiple species (e.g. mouse, rat and human) and various cell types and tissues [32,33]. It provides a valuable tool to integrate gene networks and phenotypic traits, and also allows cross-cell type and cross-species comparative gene expression and eQTL analyses. Our data can aid in the identification of candidate modulators of gene expression and/or phenotypic traits [34], and as such can serve as a starting point for hypothesis-driven research in the fields of stem cell biology and hematology.

### Materials and Methods

#### Ethics Statement
All animal experiments were approved by the Groningen University Animal Care Committee.

#### Recombinant Inbred Mice
Female BXD recombinant inbred mice were originally purchased from The Jackson Laboratory and housed under clean conventional conditions. Mice were used between 3 and 4 months of age.

#### Cell Purification
Bone marrow cells were flushed from the femurs and tibias of three mice and pooled. After standard erythrocyte lysis, nucleated cells were stained with either a panel of biotin-conjugated lineage-specific antibodies (containing antibodies to CD3ε, CD11b (Mac1), CD45R/B220, Gr-1 (Ly-6G and Ly-6C) and TER-119.

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**Table 1. Overview of static and dynamic eQTLs**

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<th>eQTL category</th>
<th>eQTL subcategory</th>
<th># probes</th>
<th># markers</th>
<th># probes/# markers</th>
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<td>2.28</td>
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RNA Isolation and Illumina Microarrays

Total RNA was isolated using the RNeasy Mini kit (Qiagen) in accordance with the manufacturer’s protocol. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion) according to the manufacturer’s specifications starting with 100 ng total RNA. Per sample, 1.5 mg of cRNA was used to hybridize to Sentrix Mouse-6 BeadChips (Illumina). Hybridization and washing were performed by ServiceXS according to the Illumina standard assay procedures. Scanning was carried out on the Illumina BeadStation 500. Image analysis and extraction of raw expression data were performed with Illumina Beadstudio v2.3 Gene Expression software with default settings and no normalization. The raw expression data from all four cell types were first log2 transformed and then quantile normalized as a single group.

Clustering of Genes

For cluster analysis we retained only genes having a minimal fold change of 2 (difference of 1 in log2 scale) in either direction in mean expression on the transition from Lin−Sca-1+ c-Kit+ to Lin−Sca-1− c-Kit+ and on the transition from Lin−Sca-1− c-Kit+ to TER-119+ or to Gr-1+. This filter reduced the dataset to 876 probes. We then computed the distance matrix for this group of probes, using the absolute Pearson correlation. Using this distance matrix, we applied the hierarchical clustering algorithm. From the resulting tree, 8 different clusters emerged from a manually chosen threshold. We then submitted each of these clusters to DAVID to identify enriched functional annotations [35].

Full ANOVA Model for eQTL Mapping

The expression data of the four cell types were firstly corrected for batch effect and then analyzed separately by the following ANOVA model:

\[ y_{ij} = \mu + Q_i + (Q_i \times CT_j) + e_{ij} \]

where \( y_{ij} \) is the gene’s log intensity on the \( i \)th microarray; \( \mu \) is the mean; \( Q_i \) is the genotype effect under study; and \( e_{ij} \) is the residual error.

Next, expression data of the four cell types were combined and analyzed by a full ANOVA model including the cell type effect (CT) and the eQTL×CT interaction effect:

\[ y_{ij} = \mu + CT_j + (Q_i \times CT_j) + e_{ij} \]

where \( y_{ij} \) is the gene’s log intensity at the \( i \)th microarray \( (i = 1, \ldots, n) \) and \( j \)th cell type; \( CT_j \) is the \( j \)th cell type effect; \( (Q_i \times CT_j) \) is the interaction effect between the \( i \)th eQTL genotype and \( j \)th cell type, and \( e_{ij} \) is the residual error. The batch effect was included as one of

Figure 3. Quantitative overview of static and dynamic eQTLs. (A) Pie charts presenting all 365 static and 1283 dynamic eQTLs that were detected with \( p<10^{-8} \). Dynamic eQTLs are subdivided in all 14 categories of interaction eQTLs. (B) Matrix showing the four cell-type-dependent dynamic eQTL categories and the cell type in which the gene was expressed most highly. (C) All static and dynamic eQTLs are subdivided in local and distant eQTLs. Shown is which number of eQTLs was detected by Illumina probes that hybridize to sequences that are known to contain polymorphisms (SNPs) between the two parental strains. Abbreviations: S, stem cells; P, progenitor cells; E, erythroid cells; M, myeloid cells.

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the factors. For each probe, we performed a genome-wide linkage analysis to identify the two markers that showed the most significant main QTL effect and interaction effect, respectively.

**Local and Distant eQTLs**

We defined an eQTL as local if it was located within less than 10 Mb from the gene. All other eQTLs were considered distant.

**Classification of eQTLs**

The ANOVA yields significance p-values for the main QTL effect $Q_i$ and the interaction effect $(Q_i \times CT)_j$ for each probe at each marker. A small p-value for the interaction effect indicates that the eQTL effect is different between the cell types. This significant difference can be due to very diverse patterns, with different biological interpretations. It is therefore necessary to classify interaction eQTLs based on these patterns. To achieve this classification, for every interaction eQTL, we evaluated the strength of the effect in each cell type by calculating the difference between the mean expression of both genotypes. The cell type for which the effect was the strongest was labeled “High.” The cell type whose effect was most different from the strongest effect was labeled “Low.” The remaining two cell types were assigned to the group they resembled most closely. This classification allowed us to define 14 categories of interaction eQTLs. Additionally, we identified eQTLs that have a consistent effect across all four cell types. This category of consistent eQTLs consists of all probes satisfying the following three conditions: the gene has a significant main effect $Q_i$ at marker $m$; for the same marker $m$, the interaction $(Q_i \times CT)_j$ is not significant; the mean eQTL effect across cell types has a coefficient of variation smaller than 0.3.

**Estimating the FDR for the Main QTL Effect**

We permuted the strain labels in the genotype data 100 times, maintaining the correlation of expression traits while destroying any genetic association. Then we applied the full ANOVA model and stored the genome-wide minimum p-value for each transcript. Based on the resulting empirical distribution of p-values, we estimated that a threshold of $-\log_{10} p = 6$ corresponds to a false discovery rate [36] of 0.02 for the main QTL effect. The 99.9th percentile of the number of significant eQTLs per marker (i.e., the minimum size of statistically significant “eQTL hotspots”) is 28.

**Estimating the FDR for Interaction QTL Effect**

We estimated the residuals of the full ANOVA model after fitting all factors up to the main QTL effect at each marker for each transcript [37]. Then we permuted the strain labels and applied the ANOVA model $y_{ij} = Q_i + CT_j + (Q_i \times CT)_j + e_{ij}$ to the permuted residuals at each marker for each transcript and stored the genome-wide minimum p-value. Based on 100 permutations and the resulting empirical distribution of p-values, we estimated that a threshold of $-\log_{10} p = 6$ corresponds to a false discovery rate of 0.021 for interacting QTL effect. The 99.9th percentile of the number of significant eQTLs per marker (i.e., the minimum size of statistically significant “interaction hotspots”) is 8.

**Detection of Swapping eQTLs**

Swapping eQTLs are those transcripts that show one eQTL in one cell type, but another eQTL in another cell type. From the full model mapping described above, we obtained 1283 transcripts with a significant interaction effect between genotype (first marker) and cell type. After taking into account the genetic and interaction effects of the first marker, we scanned the genome excluding the region of the first marker (window size = 30 cM) and tested if there was a significant interaction effect between genotype and cell type and whether this new interaction effect was classified in a different cell type category (see above Classification of eQTLs), which would indicate a swapping eQTL.

This means, for each transcript, a two-marker full model mapping was applied using the following model:

$$y_{ij} = \mu + CT_j + Q_i^* + (Q_i^* \times CT)_j + Q_i + (Q_i \times CT)_j + Q_i^* Q_i + e_{ij}$$

where $y_{ij}$ is the gene’s log intensity at the $i$th microarray ($i = 1, \ldots, n$) and $j$th cell type; $CT_j$ is the $j$th cell type effect; $Q_i$ and $(Q_i \times CT)_j$ are the main genotype effect at first marker and interaction effect between cell type and the genotype effect at this marker, where the first marker is defined as the marker with maximal interaction effect from previous one-marker full model mapping; $Q^*_i$ is the genotype effect of the second marker; $(Q_i \times CT)_j$ is the interaction effect between the $i$th genotype and $j$th cell type; $Q^*_i Q_i$ is the epistasis effect and $e_{ij}$ is the residual error.

**URLs**

All raw data were deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE18067). All processed data were deposited in the GeneNetwork (http://www.genenetwork.org) [32,33].

**Supporting Information**

**Figure S1** Analysis of the quantitative aspects of eQTLs. (A) Strain distribution pattern (expression values per strain) of H2-Ob transcript levels in stem cells. H2-Ob is a locally regulated transcript where only strains that carry the B6 allele of the gene regulated by a strong eQTL mapping within 10 Mb from the corresponding gene. We compared the strength of the genetic association (eQTL effect) with the mean expression levels of the corresponding genes. Each dot refers to a single probe. If the eQTL effect is negative, B6 alleles at the locus most strongly associated with variation in transcript abundance increase its expression. If the eQTL effect is positive, D2 alleles at the eQTL increase expression. The data are shown for stem cells, but identical patterns were obtained for the other three cell populations. (D) This plot illustrates the size of the effect of the presence of either parental B6 or D2 allele at the eQTL on gene expression levels. Each dot refers to a single probe. For each probe expression values for strains carrying the B6 allele at the strongest associated marker were compared with values for strains carrying the D2 allele. Indicated in red are transcripts that are locally regulated by a strong eQTL mapping within 10 Mb from the corresponding gene.

**Table S1** Clustering results.

**Table S2** Principal eQTL categories.

**Table S3** All dynamic eQTLs.
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References


Author Contributions

Conceived and designed the experiments: LVB RCJ GdH. Performed the experiments: AG LVB EW AA BD. Analyzed the data: AG YL BMT RB. Contributed reagents/materials/analysis tools: YL BMT XB RW RCJ. Wrote the paper: AG YL BMT RB RCJ GdH.