High-throughput identification of human SNPs affecting regulatory element activity

Joris van Arensbergen1*, Ludo Pagie1, Vincent D. FitzPatrick2,3, Marcel de Haas1, Marijke P. Baltissen4, Federico Comoglio1,5, Robin H. van der Weide1, Hans Teunissen1, Urmo Võsa6,7, Lude Franke6, Elzo de Wit1, Michiel Vermeulen3,4, Harmen J. Bussemaker2,3 and Bas van Steensel1*

Most of the millions of SNPs in the human genome are non-coding, and many overlap with putative regulatory elements. Genome-wide association studies (GWAS) have linked many of these SNPs to human traits or to gene expression levels, but rarely with sufficient resolution to identify the causal SNPs. Functional screens based on reporter assays have previously been of insufficient throughput to test the vast space of SNPs for possible effects on regulatory element activity. Here we leveraged the throughput and resolution of the survey of regulatory elements (SuRE) reporter technology to survey the effect of 5.9 million SNPs, including 57% of the known common SNPs, on enhancer and promoter activity. We identified more than 30,000 SNPs that alter the activity of putative regulatory elements, partially in a cell-type-specific manner. Integration of this dataset with GWAS results may help to pinpoint SNPs that underlie human traits.

An alternative functional readout is to insert DNA sequence elements carrying each allele into a reporter plasmid. On transfection of these plasmids into cells, the promoter or enhancer activity of these elements can be measured quantitatively. Different cell types may be used as models for corresponding tissues in vivo. Large-scale versions of this approach are referred to as massively parallel reporter assays (MPRAs), which have been applied to screen tens of thousands of SNPs. Each of these studies has yielded tens to, at most, several hundreds of SNPs that significantly alter promoter or enhancer activity. As these MPRAs have covered only a tiny fraction of the genome, it is likely that many more SNPs with regulatory impact are to be discovered.

Here, we report application of an MPR strategy with a >100-fold increased scale compared to previous efforts. This enabled us to survey the regulatory effects of 5.9 million SNPs in two different cell types, providing a resource that helps to identify causal SNPs among candidates generated by GWAS and eQTL studies. The data are available for download, and can be queried through a web application (https://sure.nki.nl).

Results
A survey of 5.9 million SNPs using SuRE. We applied our SuRE technology to systematically screen millions of human SNPs for potential effects on regulatory activity. SuRE is an MPR with sufficient throughput to query entire human genomes at high resolution and high coverage. Briefly, random genomic DNA fragments of a few hundred base pairs (bp) are cloned into a promoter-less reporter plasmid that, on transfection into cultured cells, only produces a transcript if the inserted genomic DNA fragment carries a functional transcription start site (TSS) (Fig. 1a). The transcript is identified and quantified by means of a random barcode sequence

1Division of Gene Regulation, Oncode Institute, Netherlands Cancer Institute, Amsterdam, the Netherlands. 2Department of Biological Sciences, Columbia University, New York, NY, USA. 3Department of Systems Biology, Columbia University Medical Center, New York, NY, USA. 4Department of Molecular Biology, Oncode Institute, Radboud Institute for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands. 5Department of Haematology, University of Cambridge, Cambridge, UK. 6Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. 7Estonian Genome Center, Institute of Genomics, University of Tartu, Tartu, Estonia.

*e-mail: joris.van.arenbergen@gmail.com; b.v.steensel@nki.nl
Fig. 1 | Identification of raQTLs by SuRE. a, Schematic representation of the SuRE experimental strategy. ORF, open reading frame; PAS, polyadenylation signal. Colors indicate different barcodes. SuRE yields orientation-specific activity information26 (SuRE +/− tracks, right-hand panel). b, SuRE signals from the four genomes in an example locus, showing differential SuRE activity at raQTL rs6739165, depending on the allele (A or C) present. Mb, megabase. c, SuRE activity for all fragments containing rs6739165. ND, not detected. SuRE data of + and − orientations are combined. Values on the y axis were shifted by a random value between −0.5 and 0.5 to better visualize DNA fragments with the same value. P < 2.2 × 10−16, according to two-sided Wilcoxon rank-sum test. d, Same data as in c, but only the expression value for each fragment is shown (without the addition of the random value). Red lines indicate mean values. e, Numbers of raQTLs in K562, HepG2 or both. f, Example of a locus showing differential SuRE activity for two genomes in HepG2 only. Below the SuRE tracks known transcript variants of POU2AF1 are indicated, and RNA-seq data from K562 and HepG2 (data from ref. 25).
that is unique for every insert, allowing for a multiplexed readout of millions of random DNA fragments. Importantly, because active promoters as well as enhancers generate transcripts, activity of both types of elements can be assayed quantitatively by SuRE\cite{note1}.

To survey a large cross-section of SNPs present in the human population, we chose four sequenced genomes of individuals from four different populations\cite{note1} (Fig. 1b). From each genome we generated two independent SuRE libraries that each contained ~300 million random genomic DNA fragments of 150–500 bp (Supplementary Fig. 1a and Supplementary Table 1). In these libraries a total of 2,390,729,347 unique genomic DNA fragments were sequenced from both ends, mapped to the reference genome and linked to their unique barcode. Among these fragments, 1,103,381,066 carried at least one SNP for which we identified both alleles in our libraries. These libraries enabled us to test promoter/enhancer activity of both alleles of 5,919,293 SNPs, which include 4,569,323 (57%) of the ~8 million known common SNPs (minor allele frequency (MAF) > 5%) worldwide\cite{note1}. Importantly, each SNP allele is covered by 122 different genomic DNA fragments on average (Supplementary Fig. 1b,c), which provides substantial statistical power.

We introduced these libraries by transient transfection into human K562 and HepG2 cell lines. K562 is an erythroleukemia cell line with strong similarities to erythroid progenitor cells\cite{note1}. HepG2 cells are derived from a hepatocellular carcinoma, and serve as an approximate representation of liver cells. After transfection of the SuRE libraries into each cell line we isolated messenger RNA and counted the transcribed barcodes by Illumina sequencing. Three independent biological replicates yielded a total of 2,377,150,709 expressed barcode reads from K562 cells, and two biological replicates yielded 1,174,138,611 expressed barcode reads from HepG2 cells.

Identification of thousands of SNPs with regulatory impact. From these data we first constructed strand-specific tracks of SuRE enrichment profiles for each of the four genomes (Fig. 1b). This revealed thousands of peaks that generally colocalize with known enhancers and promoters (Supplementary Fig. 1d), as reported previously\cite{note1}. For a subset of peaks, the magnitude varied between the four genomes and showed a correlation with a particular allele of a coinciding SNP. For example, in K562 cells we detected a strong SuRE signal overlapping with SNP rs6739165 in the genomes that are homozygous for the C allele, but no signal in the genome that is homozygous for the A allele and an intermediate signal in the genome that is heterozygous for this SNP (Fig. 1b).

To systematically annotate SNPs we combined the complete SuRE datasets from the four genomes for each transfected cell line. The sequencing data of the SuRE libraries then enabled us to group, for each SNP, the overlapping genomic DNA fragments by the two alleles (Fig. 1c,d). This allowed us to identify SNPs for which fragments carrying one allele produced significantly different SuRE signals compared to those carrying the other allele. Because all of these fragments differ in their start and end coordinate, the activity of each allele is tested in a multitude of local sequence contexts, providing not only statistical power but also biological robustness. For each SNP we calculated a \( P \) value and we used a random permutation strategy to estimate false discovery rates (FDR) (Supplementary Fig. 1e,f). We also required that the strongest allele showed an average SuRE signal of at least fourfold over background. We refer to the resulting SNPs at FDR < 5% as reporter assay QTLs (raQTLs).

This analysis yielded a total of 19,237 raQTLs in K562 cells and 14,183 in HepG2 cells (Fig. 1e). The average allelic fold change of these SNPs was 4.0-fold (K562) and 7.8-fold (HepG2) (Supplementary Fig. 1g,h). In 72% of cases the SuRE effect could be assigned to a single SNP; when SNPs were spaced less than ~200 bp apart, their effects could typically not be resolved (Supplementary Fig. 1i).

Most raQTLs were detected in either K562 or HepG2 cells, but not in both (Fig. 1e). The overlap may be underestimated due to the arbitrary FDR and expression cutoffs used to define these sets (Supplementary Fig. 1j). Nevertheless, many SNPs show clear cell-type-specific effects (Supplementary Fig. 1j–l). For example, rs4265625[G] creates regulatory activity in HepG2 only (Fig. 1f). This is interesting, because rs4265625 lies in POU2AF1, a gene that has been linked to primary biliary cirrhosis—a liver disease—in a GWAS\cite{note2}. In about 1,300 instances, a K562-specific raQTL and a HepG2-specific raQTL are in strong LD \((R^2 > 0.8)\). An interesting possibility is that the two raQTLs in such linked pairs may contribute to the regulation of a common target gene, but each in a cell-type-specific manner.

raQTLs are enriched for known regulatory elements. We systematically analyzed the overlap of the raQTLs with known regulatory elements in K562 cells\cite{note1}. Compared to all SNPs analyzed, raQTLs showed 5–15-fold enrichment for promoter- and enhancer-related chromatin types, and deletion for repressed or transcribed chromatin types (Fig. 2a). We also observed strong enrichment of raQTLs in DNase hypersensitive sites (DHS) (Fig. 2b,c). This is consistent with SuRE signals overlapping with enhancers or promoters, as shown previously\cite{note1}.

Some of the raQTLs are heterozygous in the genome of K562 cells. For these SNPs we investigated whether the allelic imbalance observed by SuRE was reflected in a corresponding imbalance in the DHS signal. For example, the SuRE signal at rs12985827, a non-coding SNP in an intron of the APC2 gene, has a strong bias for the C allele (the alternative allele, ALT) over the T allele (the reference allele, REF) (Fig. 2d). Indeed, it shows a similar allelic imbalance for DHS (Fig. 2e). Among the 616 raQTLs that were heterozygous in K562 and showed sufficient DNase-seq coverage, we observed a strong skew for higher DNase sensitivity at the more active allele, compared to 616 heterozygous non-raQTL control SNPs that overlapped DHSs (Fig. 2f,g). We found a similar but less pronounced trend in H3K27ac\cite{note3} and ATAC-seq\cite{note4} data from K562 cells (Supplementary Fig. 2). We conclude that available epigenomic data is generally consistent with the SuRE results.

Altered transcription factor binding sites at raQTLs. The observed changes in enhancer or promoter activity are likely explained by SNPs changing transcription factor (TF) binding motifs. For example, the T allele of rs12985827 disrupts an EGR1 binding motif and leads to reduced SuRE activity (Figs. 3a and 2d). To investigate this systematically, we made use of the computationally predicted changes in TF motifs in the SNP2TFBS database\cite{note5}. Among the set of raQTLs in K562 and HepG2 cells, 31 and 38% are predicted to alter the motif of at least one TF, respectively. This is a 1.6-fold and 1.9-fold larger proportion than for all SNPs, respectively (Fig. 3b). Moreover, 67 and 69% of the raQTLs showed concordance between the predicted effect on motif affinity and SuRE expression, that is, the allele with the weakest motif resemblance had the lowest SuRE expression (Fig. 3c).

We note that 100% concordance should not be expected in this analysis, for example, because not all TF binding motifs are known, some may be misannotated and some TFs can act as repressors.

We expected motifs in raQTLs to reflect the sets of TFs that are selectively active in the respective cell types. Indeed, raQTLs in K562 were enriched for motifs of TFs that are primarily active in the erythroid blood lineage, such as GATA and STAT factors, while raQTLs in HepG2 cells were enriched for motifs of TFs that are specific for liver cells, such as HNF factors (Fig. 3d). Disruptions of the TP53 motif also appeared to be relatively more consequential in HepG2, which is presumably related to the known inactivation of TP53 in K562 cells\cite{note1} but not in HepG2 cells\cite{note1}. Together, these data point to a general concordance between the detected changes in SuRE activity and predictions based on sequence motif analysis.
No evidence for strong negative selective pressure on raQTLs.

It has been observed that genes that do not have cis-eQTLs are more likely to be loss-of-function (LOF)-intolerant genes, possibly reflecting selection against variants acting on such genes. We found that the fraction of SNPs that are raQTLs was significantly, but only slightly, lower in the proximity of LOF-intolerant genes than in the proximity of LOF-tolerant genes (Supplementary Fig. 3a,b). However, for a set of control SNPs that were matched to the raQTLs for their SuRE activities and coverage in the SuRE libraries we observed a similar pattern (Supplementary Fig. 3a,b). This suggests that the overall density of active regulatory elements, rather than elements affected by SNPs, is lower near LOF-intolerant genes. Genome wide, we observed slightly lower MAFs for our raQTLs as compared to matched SNPs, but only for the K562 dataset and not for the HepG2 dataset (Supplementary Fig. 3c,d). This modest under-representation of raQTLs in the human population is consistent with recent computational predictions and may point to a slight negative selection pressure. Taken together, we found no evidence for strong negative selective pressure on raQTLs.

Integration of SuRE with eQTL maps.

Next, we integrated our SuRE data with eQTL mapping data from the GTEx Project. We compared SuRE data from K562 and HepG2 cells with eQTL data from the most closely related tissues, that is, whole blood and liver, respectively. Strong similarity between the two data types should not be expected, because in the GTEx data each gene with significant associations (eGene) is linked to 101 eQTL SNPs on average, of which only one or a few may be causal. Rather, we regard the SuRE data as a filter to identify the most likely causal SNPs among the large number of eQTL candidates.

For each raQTL, the log2(ALT/REF) SuRE signal may be concordant with the eQTL signal (that is, having the same sign as the slope of the eQTL analysis) or discordant (having the opposite sign). The simplest interpretation of concordance is that the SNP alters a
regulatory element that positively regulates the eGene; if an allele reduces the activity of the element then it will also reduce the activity of the eGene. Discordance may point to mechanisms that are more indirect, for example, when an SNP alters the promoter of an antisense transcript that in turn interferes with the sense expression of the eGene. In line with this interpretation, concordant raQTLs are enriched near the TSSs of the eGenes (Supplementary Fig. 4a,b). The slightly higher odds ratios for HepG2 versus liver may be due to a stronger similarity of HepG2 to liver cells than of K562 to blood cells. Because discordant effects are more difficult to interpret, we further focused on SNPs with concordant effects.

Candidate causal SNPs in eQTL maps and their putative mechanism. For several physiologically relevant eGenes we highlight the most likely causal SNPs based on our SuRE data, and we provide insights into the potential underlying mechanisms.

A first example is XPNPEP2, a gene associated with the risk of angioedema in patients treated with an angiotensin-converting enzyme inhibitor. The GTEx project has linked the expression of XPNPEP2 to a SNP as in Fig. 2d,e) and the sequence logo for EGR1. The T allele disrupts a conserved nucleotide in the EGR1 binding motif. Compared to all SNPs (n = 5,919,293), raQTLs in K562 (n = 19,237) and HepG2 (n = 14,183) both overlap preferentially with computationally predicted alterations of TF binding motifs according to SNP2TFBS, \( P < 2.2 \times 10^{-16} \), according to two-sided Fisher’s exact test. Concordance between the predicted increase or decrease in TF binding according to SNP2TFBS and the observed effect in SuRE, assuming that decreased TF binding typically leads to decreased activity of a regulatory element. \( P < 2.2 \times 10^{-16} \), according to two-sided Fisher’s exact test. TF motif alterations that are preferentially present among raQTLs in either K562 or HepG2 cells. Only the seven most enriched TF motifs for each cell type are shown.

A third example is the neighboring genes YEATS4 (encoding a transcription regulator) and LYZ (encoding lysozyme, an antibacterial protein). Overlapping sets of eQTL SNPs were identified for these genes in whole blood (Fig. 4e and Supplementary Fig. 4c). Among these, SuRE in K562 cells identified two neighboring raQTLs (rs623853 and rs554591) located ~400 bp downstream of the YEATS4 TSS, which both show concordance with the eQTL data (Fig. 4e).

To identify TFs that might be responsible for the differential SuRE activity at these two raQTLs, we conducted an in vitro binding proteomic analysis36,37. Briefly, we immobilized double-stranded oligonucleotides carrying each of the two alleles on beads, and incubated them with nuclear extract from K562 cells. After washing to remove unbound proteins, we used on-bead trypsin digestion followed by dimethyl stable isotope labeling38 and quantitative mass spectrometry to identify proteins that preferentially associated with one of the two SNP alleles. In this assay, at rs623853 the weaker A allele caused strong loss of binding of Ets-like factors (Fig. 4f), consistent with a disruption of the cognate motif (Fig. 4g). The A allele also showed moderately increased binding of several other proteins. At rs554591 the C allele caused strong loss of ZNF787 and gain of several other factors including KLF and SP proteins (Supplementary Fig. 4d). The variants of both SNPs may thus cause altered binding of TFs and their cofactors, leading to altered enhancer activity. K562 cells are heterozygous for both rs623853 and rs554591, but no significantly different DHS signal is detectable for either of the alleles (Supplementary Fig. 4e,f).

These examples illustrate that SuRE can prioritize SNPs as likely causal candidates from a set of tens to hundreds of eQTL SNP candidates. By integrating our data with the GTEx datasets, SuRE identified at least one raQTL among the eQTL SNPs for 20.0% of the 8,661 eGenes in whole blood, and for 11.1% of the 4,000 eGenes in liver.

Integrating SuRE data with GWAS data. SuRE may also help to identify candidate causal SNPs in GWAS data. We focused on a large GWAS that identified 6,736 lead SNPs and more than 1 million linked significant SNPs associated with at least one of 36 blood-related traits39. These SNPs occurred in LD clusters, each on average

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**Fig. 3 | Concordance of SuRE data and predictions based on TF binding motifs.** a. Comparison of the sequence flanking raQTL rs12985827 (same SNP as in Fig. 2d,e) and the sequence logo for EGR1. The T allele disrupts a conserved nucleotide in the EGR1 binding motif. b. Compared to all SNPs (n = 5,919,293), raQTLs in K562 (n = 19,237) and HepG2 (n = 14,183) both overlap preferentially with computationally predicted alterations of TF binding motifs according to SNP2TFBS, \( P < 2.2 \times 10^{-16} \), according to two-sided Fisher’s exact test. c. Concordance between the predicted increase or decrease in TF binding according to SNP2TFBS and the observed effect in SuRE, assuming that decreased TF binding typically leads to decreased activity of a regulatory element. \( P < 2.2 \times 10^{-16} \), according to two-sided Fisher’s exact test. d. TF motif alterations that are preferentially present among raQTLs in either K562 or HepG2 cells. Only the seven most enriched TF motifs for each cell type are shown.
consisting of 158 SNPs and represented by a single statistically most significant (lead) SNP. The lead SNPs are not necessarily the causal SNPs, but are more likely to be the causal SNPs or in strong LD with the causal SNP(s). We therefore searched within a 100-kb window from each lead SNP for overlap between significant GWAS SNPs and raQTLs. For 1,238 out of 6,736 lead SNPs this yielded at least one linked raQTL. These raQTLs were preferentially located close to the lead SNPs, compared to a set of matching control SNPs (Fig. 5a). Overall, the enrichment of SuRE raQTLs among the total set of GWAS SNPs did not significantly exceed that of the matching
control set of SNPs (1,188), but this was to be expected considering that only one or a few of the, on average, 158 significant SNPs may be true causal SNPs.

One example where SuRE provided a clear candidate among the GWAS SNPs is rs4572196, which is within 100 kb of 11 lead SNPs associated with various mature red blood cell traits, such as 'hemoglobin concentration' and 'hematocrit'\(^{44}\). In none of the 11 GWAS associations is rs4572196 the lead SNP, but in SuRE the G allele shows an approximately eightfold higher activity than the A allele (\(P = 2.0 \times 10^{-8}\)) and it is the only SNP in the region with a \(P\) value below our cutoff (Fig. 5b). By in vitro proteomics we identified several proteins with differential binding activity to the two rs4572196 alleles. JUN proteins showed stronger binding to the G allele (Fig. 5c), as one might predict based on the JUN binding motif (Fig. 5d). The GWAS demonstrated a positive association between the reference A allele and hemoglobin concentration and red blood cell counts\(^{46}\). Interestingly, rs4572196 lies \(\sim 11\) kb upstream of \(SH2B3/\)LNK, a gene that inhibits erythropoiesis in mouse\(^{47}\). SuRE identifies the A allele as the weak allele, potentially reducing SH2B3 expression. We cannot rule out that other SNPs in the region, for example, among those not included in our SuRE data, also play a causal role.

Another example is rs3748136, which, together with 66 other SNPs in this locus, was found to be linked to blood counts of reticulocytes. Among the 59 SNPs covered in our data, rs3748136 is the only significant SuRE hit, with the A allele showing an \(\sim 18\) -fold higher activity than the G allele (\(P = 7.5 \times 10^{-20}\)) (Fig. 5e). K562 cells are heterozygous for this SNP and, indeed, show a strong allelic imbalance in DHS-seq, with the A allele being the more active allele (Fig. 5f). In vitro binding proteomic analysis identified JUN and BACH1 proteins as more strongly bound to the A allele (Fig. 5g), consistent with the G allele disrupting predicted binding motifs for both proteins (Fig. 5h). Both BACH1 and JUN proteins are highly expressed in whole blood and in K562 cells\(^{48}\). Reanalysis of chromatin immunoprecipitation (ChIP) data for BACH1 (ref. 49) showed a complete allelic imbalance for BACH1 binding and the same was found for JUND (Fig. 5i). QTL analysis of whole blood\(^{1}\) has linked the A allele of rs3748136 to elevated expression of the nearby non-coding RNA gene \(NR_\text{125431}\) (Fig. 5k). To further test this, we modified the G allele in K562 cells to an A allele by CRISPR-Cas9 editing\(^{50}\). We found that expression of \(NR_\text{125431}\) in K562 cells shows considerable clone-to-clone variation (Supplementary Fig. 5a–c).

To account for this we performed G to A substitution in a stable K562 clone. This modification led to a fourfold upregulation of \(NR_\text{125431}\) (Fig. 5i).

Finally, we overlaid the HepG2 SuRE data with a recent GWAS that linked SNPs to the risk of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), a type of cancer prevalent in Asia\(^{52}\).

This study identified 61 candidate SNPs in an \(\sim 200\)-kb region that includes the \(HLA\) class I locus. Of the 50 SNPs covered by our SuRE data, two are raQTLs in HepG2 (Fig. 6). These raQTLs are intergenic; both ALT alleles show reduced SuRE signals and are associated with higher HCC risk. Besides the \(HLA\) genes that are important for antigen presentation, other genes in this region could be affected by these raQTLs and play a role in HCC. For example, \(ZNDR1\) and its antisense transcript have been implicated in expression of HBV mRNA and proliferation of HBV-infected HepG2 cells\(^{53}\).

**Discussion**

By surveying 5.9 million SNPs from four entire human genomes we identified about 30,000 SNPs that alter regulatory activity of enhancers or promoters. The data are available for download, and can be queried through a web application (https://sure.nki.nl).

Because 90% of these raQTLs were identified in only one of the two tested cell lines, it is likely that extension of this survey to other cell types will increase the number of raQTLs substantially. It is thus conceivable that several percent of all human SNPs may have an impact on the activity of regulatory elements in at least one cell type.

While the redundant design of SuRE increases the odds that a robust and biologically representative measure of SNP effects is obtained, we note that SuRE signals arising from enhancers are generally weaker than those from promoters, and it cannot be ruled out that certain enhancers cannot be detected by SuRE at all. Thus, our approach may be better powered to detect effects of SNPs on promoters compared to enhancers. Furthermore, SuRE infers effects of SNPs on enhancer activity indirectly, by virtue of the ability of most enhancers to act as autonomous TSSs. Although this feature generally correlates with the potency of enhancers to activate a cis-linked promoter, this correlation is not perfect\(^{1}\) and, in some enhancers, both activities may be differentially affected by particular SNPs.

Like most other MPRAs, SuRE queries all DNA elements in a plasmid context and in cultured cell lines, which may yield different results compared to a proper genomic context and tissue context. Integration with multiple orthogonal datasets can help provide confidence in the relevance of candidate SNPs. Epigenomic data, sequence motif analysis and in vitro binding mass spectrometry\(^{54,55}\) can serve this purpose, and, in addition, provide key insights into the mechanisms of action.

We foresee several additional applications of these SuRE data. First, there are many other eQTL studies and GWAS that may be overlaid with the SuRE maps. Second, in addition to SNPs, small insertions and deletions (indels) may be analyzed. While, in human genomes, such indels occur at an \(\sim 20\)-fold lower frequency than SNPs\(^{1}\), their individual regulatory impact may be more potent, as they tend to disrupt TF binding motifs more dramatically. Third, our datasets may be useful for studying the regulatory grammar of

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**Fig. 5** Candidate causal SNPs identified by SuRE among large sets of GWAS SNPs. a. Distribution of distances between lead SNPs for blood traits\(^{39}\) and raQTLs (black) and a set of matched control SNPs (gray). \(P\) value was obtained with a two-sided Wilcoxon rank-sum test. raQTLs in K562 cells are modestly enriched near blood GWAS lead SNPs. b. Overlay of SuRE and GWAS data for a cluster of GWAS SNPs linked to hemoglobin concentration\(^{46}\). SuRE data in K562 cells. Top and bottom end of each bar indicate the SuRE signal of the strongest and weakest alleles, respectively. Color of the bars indicates which allele is stronger. Width of the bars is proportional to -log\(_{10}(P\) value\)) (top). Positions of significant GWAS SNPs with the associated –log\(_{10}(P\) values\)) on the y axis (middle). Gene annotation (dark red: \(SH2B3/\)LNK) and DNase-seq data from K562 cells\(^{48}\) (bottom). c. Protein binding analysis, as in Fig. 4f, for rs4572196. d. Sequence of the probes used in c aligned to sequence logo for JUNB. e. Same as b but for a cluster of SNPs associated with reticulocyte counts by GWAS\(^{44}\). f. Fraction of reads containing each of the two alleles of rs3748136 in K562 genomic DNA and K562 DNase-seq reads. P value was obtained with a two-sided Fisher’s exact test. g. Same as c but for rs3748136. h. Sequence of the probes used in g aligned to binding motifs for JUNB and BACH1. i. Fraction of reads containing each of the two alleles of rs3748136 in K562 genomic DNA (left) and K562 ChIP-seq reads for BACH1 (right). j. Same as i but for ChIP-seq reads for JUND. ChIP data are from (ref. 51). k. Association between alleles of rs3748136 and \(NR_\text{125431}\) expression in whole blood according to GTEx\(^{5}\). Red lines indicate medians. L. Expression of \(NR_\text{125431}\) in subclones derived from K562 clone BL-2 subjected to CRISPR/Cas9 editing of rs1053036. Sixteen unaltered subclones and 11 \(G>A\) edited subclones were assayed by RT-qPCR of \(NR_\text{125431}\) (normalized to GAPDH). \(P\) value was obtained with a two-sided Wilcoxon rank-sum test. Red lines indicate medians. One \(G>A\) subclone appeared to have reverted to the completely inactive state seen in many K562 clones initially derived from the cell pool (Supplementary Fig. 5c).
TFs, as they cover natural genetic variation in thousands of regulatory elements. For example, the SuRE data may be used to refine computational predictions of SNP effects. Finally, it will be interesting to expand this type of analysis to individuals with a genetic disorder to capture additional disease-relevant variants that might not be found in the general population.
Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0455-2.

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Author contributions

J.v.A. designed and performed experiments, analyzed data and wrote the manuscript. L.P., V.D.F. and H.J.B. developed algorithms and analyzed data. M.d.H. M.P.B., M.V., R.H.v.d.W., H.T., E.d.W., U.V., E.d.W. and L.F. generated and/or analyzed data. F.C. developed the web application. B.v.S. designed experiments, analyzed data and wrote the manuscript.

Competing interests

J.v.A. is founder of Gen-X B.V. (http://www.gen-x.bio/). E.d.W. is co-founder and shareholder of Cergentis B.V. F.C. is a co-founder of enGene Statistics GmbH.

Additional information

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Correspondence and requests for materials should be addressed to J.v.A. or B.v.S.

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SuRE library preparation and barcode-to-fragment association. SuRE libraries were generated as described previously. DNA was isolated from lymphoblast cell lines Hg02601, GM18983, HG01241 and HG03464 obtained from Coriell Institute, fragmented and gel-purified to obtain ~300 bp elements. For each genome, two SuRE libraries were generated, each of an approximate complexity of 300 million fragment–barcode pairs. This was done by transformation in CloneCatcher DHSG electrocompetent Escherichia coli cells (Genlantis, catalog no. C810111), or in E. coli 10G cells (Lucigen, catalog no. 60107-1). Barcode-to-fragment association was done as described previously, except that because of the smaller genomic insert size no digest with a frequent cutter was required. Thus, after I-CeuI digest and self-ligation we immediately proceeded to the I-SceI digest.

Cell culture and transfection for SuRE. K562 cells were cultured and transfected as described. HepG2 (ATCC, catalog no. HB 8065) were cultured according to the supplier’s protocol and transiently transfected in the same manner as K562, except that program T-028 was used for nuleofection, 7.5ug of plasmid was used for each 5 million cells and cells were collected 48h after transfection. One hundred million cells were transfected for each replicate. Every 3 months all cells in culture were screened for mycoplasma using PCR (Takara, catalog no. 6601).

Illumina sequencing. Paired-end (PE) sequencing (150 bp) of SuRE libraries was done by Novogene on the HiSeqX platform, generating about 1 billion reads per library. Standard full genome sequencing and allele calling for the K562 cell was done by Novogene on the HiSeq-X platform, generating about 1 billion reads Illumina sequencing.

Sequencing data processing. PE reads of the SuRE libraries (for associating genomic positions and barcodes for each SuRE fragment) and single-end reads (SE reads) of the PCR-amplified barcodes (representing raw SuRE expression data) were processed to remove adapter and vector backbone sequences, using cutadapt (v1.9.1) PE and SE reads were discarded if the barcode sequence contained Ns or the sequence was not exactly 20 nucleotides. The remaining sequences in the PE reads are combinations of barcode sequences and genomic DNA sequences, whereas the SE reads only yield barcode sequences. The latter barcodes are simply recorded and counted. The genomic DNA sequences of the SuRE libraries were mapped to the reference genome sequence (hg19, including only chr1–22, chrX), using bowtie2 (v.2.3.2) with a maximum insert length set to 1 kb. Read pairs with either the forward or the reverse genomic DNA sequence less than six nucleotides, and read pairs not aligned as ‘proper pair’, were discarded. To prevent allelic biases in alignment we used WASP and SNP annotations from the 1000 Genome Project (Supplementary Table 3, external data source 17) to discard all reads potentially resulting in biased alignments.

The resulting associations of barcode sequence–genomic position pairs were further processed as follows:

(1) Identical barcodes associated with multiple alignment positions were discarded except for the most abundant barcode–position pair.

(2) Different barcodes associated with the exact same alignment position were merged; that is, the barcode sequence associated with this position was set to the most frequent barcode sequence in the set, and the total number of PE reads in the set was used as count for this barcode–position pair.

Next, the barcodes identified in the SE reads were matched to the barcodes in the remaining barcode–position pairs, and SuRE count tables were generated associating barcode sequences, genomic positions and counts for associated PE reads and matched SE reads for each of the biological replicates.

SNP annotation. The fragments, specified in the SuRE count tables were further annotated with SNP positions and base identities. For this annotation only SNPs were considered that were single-nucleotide, bi-allelic and the alternative allele in all four genomes were discarded. For each SNP in such a fragment we determined its base identity as observed in the actual sequence reads. Some fragments are too long to be entirely covered by the PE reads. In these cases the unidentified SNPs were assigned the IUPAC representation of both alleles; if the two alleles were known to be identical in the genome then that was used to construct the particular library (based on annotation by the 1000 Genome Project; Supplementary Table 3, external data source 17), this inferred allele was used for annotation. At the time of finalizing this article we noticed that, due to a minor coding error, ~0.4% of the SuRE fragments were incorrectly annotated to carry both the REF and ALT allele of an SNP. This may cause a very slight underestimate of the total number of raQTLs, but it does not alter any of the reported conclusions.

SuRE data analysis and visualization. Data analysis and figure production was mostly done using various R (https://www.R-project.org) and BioConductor packages.
we retrieved the DNase-seq allele counts for the 2,500 overlapping SNPs with the highest SuRE P values. We required at least 20 DNase-seq reads covering the SNP and from the resulting set we randomly selected a subset of SNPs of the same size as the set of raQTLs. We called ‘conditionally independent cis-variant associations’ in this control set as we did to the raQTLs. The comparison in Supplementary Figure 2 with H3K27ac (Supplementary Table 3, external data source 20 and 21) and ATAC-seq (Supplementary Table 3, external data source 22 and 23) was done in the same manner as that described for the DNase-seq data, except that we required only ten reads to cover the SNP, since these data are of approximately 5–10 times lower sequencing depth. We excluded a third ATAC-seq replicate (GSM2695562), since for this replicate, we did not observe a similar pattern of enrichment as for the other ATAC-seq replicates. The mean enrichment profiles shown in Supplementary Figure 2c–e were generated with Bioconductor package CoverageView (v.1.4.0).

Re-mapping of BACH1 and JUND ChIP-seq data. Fastq files were downloaded from the SRA repository (Supplementary Table 3, external data sources 13 and 14) using fastq-dump from the SRA-tools package (v.2.2.8, https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/). For BACH1 we downloaded data from datasets SRR502556 and SRR502557, and for JUND from datasets SRR502562 and SRR502543. Reads were aligned to the genome using the reference sequence hg18, including chr1–22, chrX), using bowtie2 (v.2.3.2)48 with default settings. For the comparison of motif disruptions in K562 and HepG2 we identified all raQTLs of K562 and HepG2 that caused motif disruptions, we in motif score). For the comparison of motif disruptions in K562 and HepG2 SRR502542 and SRR502557 and for JUND from datasets SRR502542 and SRR502543. Reads were aligned to the genome using the reference sequence hg18, including only chr1–22, chrX), using bowtie2 (v.2.3.2)48 with default settings.

Allele frequencies. MAFs of SNPs were obtained from the 1000 Genomes Project (Supplementary Table 3, external data source 17). Common SNPs were defined as SNPs with MAF > 0.01. When the SuRE effect could be resolved to a single SNP was calculated as the fraction of raQTLs for which neither neighbor SNP was also a raQTL. The window of 100 kb was chosen to be substantially larger than the typical size of an LD block. For these lead SNPs we calculated the distance to SNP with the lowest 100 kb of each of the 6,736 lead SNPs identifying 1,238 lead SNPs within 100 kb of

CRISPR/Cas9-mediated editing of rs3748136. We performed our CRISPR experiments on a K562 subclone in which NR_125431 was active (subclone BL_2) because initial experiments revealed that in the K562 pool, NR_125431 is expressed in only ~25% of the cells (Supplementary Fig. 5c). Five million of the BL_2 cells were nucleoected as described above with 2 μg of vector pX330-U6-Chimeric_BB-CBh-hSpCas9, a gift from F. Zhang (Addgene, plasmid no. 42230)49, and after 48 h, the genomic DNA was extracted with the QiAamp DNA Mini Kit (Qiagen) and stored at −20 °C until use. For genotyping we used a PCR amplicon that included SNP rs453301, ~250 bp downstream of rs3748136 that was also heterozygous in K562. After performing editing efficiency for the population of cells using Sanger sequencing and TIDE analysis50, single cells were cloned out. After expansion, clones were genotyped on the single PCR amplicon, and classified as successfully edited when they were heterozygous (that is, not edited) at rs453301 but homozygous for rs3748136, or that the third of the clonal population was homozygous for rs3748136. As a control we did the same procedure for the chromosome that was edited at rs3748136 was the only chromosome showing expression of NR_125431 to begin with, we also did the same at the increased expression from that chromosome after editing (see main text), even though the RT–qPCR is not allele specific. See Supplementary Table 2 for oligonucleotide sequences used.

Targeted locus analysis. To determine the phasing of the K562 genome around rs1053036, targeted locus analysis was performed essentially as described51. Briefly, roughly 5 million K562 cells were cross-linked with 4% formaldehyde and cut with NaiII. After ligation, the template was de-cross-linked and further digested with NspI. The second ligation yields circular DNA, which is used as input for the inverse PCR reaction. We performed two PCR reactions: the first with primers adjacent to rs1053036, located in the last exon of NR_125431 and the second with primers adjacent to rs3748136, located in the intergenic region (Supplementary Table 2). The PCR amplicons were combined and we generated sequencing libraries using the KAPA High Throughput Library Preparation Kit (Roche, catalog no. 7961901001). We generated 2×150-bp PE sequences on an Illumina MiSeq. Sequence reads were mappeld to hg19 using BWA SW52. The resulting bam files and the K562 vcf file (obtained from whole-genome sequencing at Novogene) were used as input for HapCUT2 (ref. 53) with the –hc option turned on to phase the alleles.

RT–qPCR. RNA was isolated from 1–5 million cells using Trizol (Bioline, catalog no. 10103036). DNA digestion was performed on ~1.5 μg of RNA using 0.5 units of DNase I for 30 min (Roche, catalog no. 0471628001) and DNA lase was inactivated by addition of 1 μl of 25 mM EDTA and incubation at 70 °C for 10 min. cDNA was produced by adding 1 μl of 50 ng/μl random hexamers and 1 μl of dNTPs (10 mM each) and incubated for 5 min at 65 °C. Then, 4 μl of first strand buffer, 20 units of RNase inhibitor (Promega, catalog no. E04001), 1 μl of reverse transcriptase (Bioline, catalog no. BIO-65501) and 2 μl of water were added and the reaction mix incubated for 10 min at 25 °C followed by 45 min at 45 °C and heat inactivation at 85 °C for 5 min. Quantitative PCR (qPCR) was performed on the Roche LightCycler480 II using the Sensifast SYBR No-ROX mix (Bioline, catalog no. BIO-98020). All expression levels were calculated using the 2−ΔΔCt method and normalized to the internal control GAPDH. See Supplementary Table 2 for oligonucleotide sequences used for qPCR.

DNA affinity purification and liquid chromatography–mass spectrometry (LC–MS) analysis. Nuclear extracts were generated from K562 cells essentially as described54. Briefly, cells were washed with PBS and then resuspended in 5% cell pellet volumes of hypotonic buffer A (10 mM HEpes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 10 mM KCl). After incubation for 10 min at 4 °C, cells were collected by centrifugation and resuspended in two pellet volumes of buffer A supplemented with 0.15% NP-40. Cells were then lysed by dounce homogenization using 35 strokes with a type B (tight) pestle on ice. Crude nuclei were collected by centrifugation then lysed in two pellet volumes of buffer C (420 mM NaCl, 20 mM HEPES pH 7.9, 20% (v/v) glycerol, 2 mM MgCl2, 0.2 mM EDTA, 0.1% NP-40, EDTA-free complete protease inhibitors (Roche) and 0.5 mM DTT) by rotation for 1 h at 4 °C. After centrifugation for 20 min at 21,000g, nuclear extract was collected as the soluble fraction. This extract was aliquoted, snap-frozen and stored at −80 °C until further use.
Oligonucleotides for the DNA affinity purifications were ordered from Integrated DNA Technologies with the forward strand containing a 5'-biotin moiety (see Supplementary Table 2). DNA affinity purifications and on-bead trypsin digestion were performed on 96-well filter plates essentially as described37. Tryptic peptides from SNP allele pull-downs were desalted using Stage (stop and go extraction) tips and then subjected to stable isotope dimethyl labeling on the Stage tips38. Matching light and heavy peptides were then combined and samples were finally subjected to LC–MS and subsequent data analyses using MaxQuant56 and R, essentially as described43.

**Statistics.** For the identification of raQTLs we tested the difference in SuRE expression of fragments containing the reference allele and the fragments containing the alternative allele using a two-sided Wilcoxon rank-sum test (see also section ‘Identification of raQTLs’). In addition, the same two-sided Wilcoxon rank-sum test was applied once after random shuffling of fragments among the two alleles. Using all obtained P values from the real comparisons and the shuffled comparisons, we estimated the FDR. In Fig. 2a, a two-sided Fisher’s exact test was performed, yielding \( P < 2.2 \times 10^{-16} \) for each of the comparisons. The number of raQTLs overlapping with each of these states was CTCF: 667; enhancer: 1,052; promoter flanking: 74; repressed: 11,024; transcribed: 1,414; transcription start site: 2,189; weak enhancer: 576. For comparisons of alleles observed for DNA sequencing and DNase-seq, ChIP-seq or RNA-seq in Figs. 2e and 5f,i,j and Supplementary Figs 4e,f and 5b, P values were obtained using a one-side Fisher’s exact test. The P values in Fig. Sb,c were obtained using a two-sided Fisher’s exact test. For the comparison in Fig. 5a, a two-sided Wilcoxon rank-sum test was done to compare the distances to the lead SNP for the raQTLs with the distances of the matched SNPs to the lead SNP.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Raw SuRE sequencing data are available at GEO (https://www.ncbi.nlm.nih.gov/geo) under accession GSE128325. SuRE count tables, BigWig files for visualization of SuRE data tracks in genome browsers, lists of raQTLs and a table with SuRE data for all 5.9 million SNPs are available from the Open Science Framework (https://osf.io/w5bzq/wiki/home/?view). SuRE data can also be queried and visualized at https://sure.nki.nl. URLs to external data sources are listed in Supplementary Table 3.

**Code availability**

Scripts are available on https://github.com/vansteensellab/SuRE-SNV-code. Software used is described in the relevant methods section and in the Nature Research Reporting Summary.

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Software and code

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**Data collection**

Paired end reads (PE reads) of the SuRE libraries (for associating genomic positions and barcodes for each SuRE-fragment), and single end reads (SE reads) of the PCR amplified barcodes (representing raw SuRE expression data), were processed to remove adapter and vector backbone sequences, using cutadapt (V1.9.1). The gDNA sequences of the SuRE libraries were mapped to the reference genome sequence (hg19, including only chr1-22, chrX), using bowtie2 (V2.3.2). To prevent allelic biases in alignment we used WASP. Full genome sequencing by Novogene of the K562 genome uses the GATK pipeline to call variants.

**Data analysis**

Data analysis and figure production was mostly done using various R (https://www.R-project.org) and BioConductor packages. Scripts are available on https://github.com/vansteensellab/SuRE-SNV-code

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Raw sequencing data are available at GEO accession GSE128325. SuRE count tables and a table with processed data for all 5.9 million SNPs is available from OSF https://osf.io/w5bzq/wiki/home/?view

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Life sciences study design

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| Sample size | Complexities of the libraries were chosen to provide good coverage for each genome |
|-------------|-----------------------------------------------------------------------------------|
| Data exclusions | No data was excluded, except for some sequencing reads for samples that were too deeply sequenced. These were downsampled; see methods. |
| Replication | We looked at correlation of SuRE expression at promoters, assuming most will not be affected by variants, and found good correlation between individuals |
| Randomization | We do not control the classification of DNA fragments as REF or VAR |
| Blinding | No blinding was used since assignment as REF or VAR alleles is done computationally. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☐ | Palaeontology |
| ☐ | Animals and other organisms |
| ☒ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
lymphoblast cell lines HG02601, GM18983, HG01241 and HG03464 obtained from Coriell Institute, HepG2 (#HB 8065; ATCC), K562 (ATCC, #CCL-243)

Authentication
Only confirmed for lymphoblast cell lines. SuRE data provides genome-wide genome sequence.

Mycoplasma contamination
All were found negative during our regular (~ every 3 months) checks

Commonly misidentified lines
None

(See ICLAC register)