rs953413 Regulates Polyunsaturated Fatty Acid Metabolism by Modulating ELOVL2 Expression

HIGHLIGHTS

- rs953413 resides in an evolutionarily conserved enhancer region
- rs953413 mediates the cooperative binding of FOXA and HNF4α to the enhancer region
- The rs953413 locus plays a key role in regulating ELOVL2 expression
- rs953413 is implicated in PUFA metabolism by regulating ELOVL2 expression
rs953413 Regulates Polyunsaturated Fatty Acid Metabolism by Modulating ELOVL2 Expression

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SUMMARY

Long-chain polyunsaturated fatty acids (LC-PUFAs) influence human health in several areas, including cardiovascular disease, diabetes, fatty liver disease, and cancer. ELOVL2 encodes one of the key enzymes in the in vivo synthesis of LC-PUFAs from their precursors. Variants near ELOVL2 have repeatedly been associated with levels of LC-PUFA-derived metabolites in genome-wide association studies (GWAS), but the mechanisms behind these observations remain poorly defined. In this study, we found that rs953413, located in the first intron of ELOVL2, lies within a functional FOXA and HNF4α cooperative binding site. The G allele of rs953413 increases binding of FOXA1/FOXA2 and HNF4α to an evolutionarily conserved enhancer element, conferring allele-specific upregulation of the rs953413-associated gene ELOVL2. The expression of ELOVL2 was significantly downregulated by both FOXA1 and HNF4α knockdown and CRISPR/Cas9-mediated direct mutation to the enhancer element. Our results suggest that rs953413 regulates LC-PUFAs metabolism by altering ELOVL2 expression through FOXA1/FOXA2 and HNF4α cooperation.

INTRODUCTION

LC-PUFAs, including ω-3 and ω-6, are essential fatty acids to mammals that cannot be synthesized de novo. The ω-6 arachidonic acid, ω-3 eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are obtained through the diet either directly or as their precursors, linoleic acid and alpha-linolenic acid. Bioconversion of LC-PUFAs from their precursors is mediated by desaturases encoded by fatty acid desaturase 1 (FADS1) and FADS2 in the FADS gene cluster and elongases encoded by ELOVL fatty acid elongase 2 (ELOVL2) and ELOVL5 (Nakamura and Nara, 2004; Zhang et al., 2016). Disrupting any of the desaturases and elongases blocked the in vivo synthesis of LC-PUFAs, which significantly affected normal growth and development in mouse models (Fan et al., 2012; Moon et al., 2009; Stroud et al., 2009; Zadravec et al., 2011). Recently, the ELOVL2 enzyme, in addition to FADS2, was verified to be another key enzyme in the synthesis of DHA in vivo (Gregory et al., 2011; Pauter et al., 2014, 2017). SNPs in the ELOVL2 locus are strongly associated with levels of LC-PUFA-derived metabolites in blood in many GWAS and meta-analyses (Table S1). Minor alleles of SNPs in the ELOVL2 locus were associated with higher levels of EPA and DPA and lower levels of DHA in plasma phospholipids, suggesting carriers of the minor alleles have lower efficiency in the bioconversion cascade from EPA to DPA and DHA catalyzed by the ELOVL2 enzyme (Lemaitre et al., 2011; Suhre et al., 2011). The identification of genetic determinants of plasma and tissue lipid levels is a key enabling factor for successful future personalized medicine interventions with EPA/DHA supplements (Chilton et al., 2017). The recent successful outcome of REDUCE-IT illustrates the value of evaluating the effects of EPA/DHA treatment on specific patient segments (hypertriglyceridemia and related lipoprotein abnormalities selected biochemically or genetically) with elevated risk that is believed to be at least partly attributable to an elevated level of the target of the intervention (Bhatt et al., 2019). The identification of functional variants behind GWAS is hindered by the linkage disequilibrium (LD) in associated regions. To prioritize the variants in LD with reported SNPs in GWAS for functional validation, we developed an allele-specific (AS)-SNPs pipeline (Cavalli et al., 2016b). This pipeline utilizes the available chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) data from the Encyclopedia of DNA Elements (ENCODE) project to detect variants located in regulatory regions and that showed allelic imbalance in chromatin binding by different transcription factors (TFs) (The Encode Project Consortium, 2012).
Liver is the most important organ for LC-PUFAs synthesis and ELOVL2 is also highly expressed in the liver. We hypothesized that the observed association of LC-PUFA profiles in blood to the ELOVL2 locus is mainly reflecting the differences in LC-PUFAs metabolism in the liver. Among all the AS-SNPs identified in human liver HepG2 cells, rs953413 located in the first intron of ELOVL2 showed significant allelic imbalance in chromatin binding. In the present study, we found that rs953413, located in an evolutionarily conserved enhancer element, directly regulates ELOVL2 expression by mediating the cooperative binding between FOXA and HNF4α factors and that it is an important contributor to the observed difference in LC-PUFAs levels among carriers of different alleles in GWAS.

RESULTS

Prioritization of Candidate Regulatory Variations in the ELOVL2 Locus

The reported SNPs in the ELOVL2 locus span a region of ~90kb that totally covers the gene body of ELOVL2 and are observed to be in high LD in the European populations (Figure S1). The alleles of the reported SNPs in LD with the major G allele of rs953413 were generally associated with a more efficient synthesis of LC-PUFAs from their precursors, e.g. synthesis of DHA from EPA and DPA (Draisma et al., 2015; Illig et al., 2009; Lemaitre et al., 2011; Li et al., 2018; Suhre et al., 2011). To pinpoint the true causal variants in the ELOVL2 locus, fine mapping of variants in this region to a 99% credible set was carried out in a trans-ethnic meta-analysis in Chinese- and European-ancestry populations (Hu et al., 2016). A total of 123 potential regulatory variations were identified in the HaploReg database that are in LD (r² > 0.8 in 1,000 Genomes Pilot I) with the SNPs in the credible set led by rs3798713 for EPA, rs9393915 for DPA, and rs953413 for DHA (Hu et al., 2016). As liver is the most important organ for in vivo synthesis of LC-PUFAs, we hypothesized that the observed association of LC-PUFA profiles in blood to the ELOVL2 locus is mainly reflecting the differences in LC-PUFAs metabolism in the liver. Only the potential regulatory variants that are annotated to be located in active promoter or enhancer regions in the liver that consist of 47 variations were kept for further consideration (Figure 1A; detailed information on variations is listed in Table S2).

To further prioritize the identified regulatory variants, we intersected the 47 regulatory variants with SNPs in the ELOVL2 locus that showed allelic imbalance in chromatin binding identified by the AS-SNPs pipeline in HepG2 cells. A total of three SNPs in the ELOVL2 locus including rs953413, rs3798713, and rs17675073 were identified to be AS-SNPs in HepG2 cells that showed allelic imbalance in chromatin binding for FOXA1 and FOXA2 (Table S3) (Cavalli et al., 2016a). As both rs953413 and rs3798713 were identified to be lead SNPs in the calculated credible set and showed allelic imbalance in chromatin binding for FOXA1 and FOXA2, they were selected for further functional validation.

rs953413 Resides in a Liver-Specific Enhancer

The ELOVL2 locus has copy number variation (CNV) in HepG2 cells, which has two copies of chromosome bearing the G allele of rs953413 (Lopez-Terrada et al., 2009; Zhou et al., 2018). To verify that the observed allelic imbalance in rs953413 and rs3798713 is not only caused by the CNV and to identify candidate causal TFs, we employed a more stringent strategy that filtered out PCR duplicates and tolerated no mismatch other than the SNP itself to each TF that mapped to these two regions in ChIP-seq experiments in HepG2 cells. Among all the TFs that have peaks overlapping or nearby rs953413 and rs3798713, only FOXA1 and FOXA2 showed reproducible allelic imbalance in chromatin binding with a preference for the G allele of rs953413 (Table S3). In accordance with occupation by multiple TFs, both the rs953413 region and rs3798713 region are highly enriched with H3K27ac signals and are also located in active DNase I hypersensitivity sites in HepG2 cells (Figure 1A). Additionally, both the rs953413 region and rs3798713 region are observed to directly interact with the promoter region of ELOVL2 identified by Capture Hi-C in GeneHancer, suggesting that these regions may be actively involved in ELOVL2 regulation (Figure 1A) (Fishilevich et al., 2017).

We next performed luciferase assay to test the enhancer activity of the two selected regions. As rs17675073 is in proximity to rs3798713, we amplified a 287-bp fragment encompassing both SNPs and inserted upstream of the minimal promoter (MP) to test its ability to drive luciferase expression. This region did show enhancer activity compared with the control plasmid without insertion. However, neither of the SNPs showed significant differences between alleles conditional on the other SNP (Figure S2). This region was then discarded for further analysis. For the rs953413 region, the enhancer activity was tested with a 529-bp fragment with rs953413 located in the middle. Both alleles of rs953413 were observed to have strong enhancer activity in cooperation with the MP in driving luciferase expression. The G allele of rs953413
had a significantly higher enhancer activity compared with the A allele (Figure 1B). This difference in enhancer activity between alleles of rs953413 was further validated with the SV40 promoter in the other luciferase construct (Figure 1C).

The meta-analysis carried out in the CHARGE Consortium provided us with the comprehensive association results for ω-3 fatty acids in plasma phospholipid in the ELOVL2 locus (Lemaitre et al., 2011). rs953413 and
Figure 2. Fine Mapping of the ME Element of the rs953413 Region to an Evolutionarily Conserved Region that Is Enriched with Putative Binding Sites for Both FOXA and HNF4α

(A) The ME element is fine mapped to an evolutionarily conserved region encompassing rs953413 (Fragment 4) by luciferase assay with a series of truncation and deletion constructs from the original 529-bp fragment. The Conservation track showed the phastCons score for 100 species from the ENCODE project.
Figure 2. Continued

(B) The ME element was predicted to have multiple conserved binding sites for both FOXA and HNF4α. The basewise conservation score for 100 species from the ENCODE project is displayed on top of the sequence. The luciferase constructs with mutations introduced into the predicted motif sequences are generated by site-directed mutagenesis and listed at the bottom. The introduced mutations for each mutation construct are listed as lower-case letters with dash line standing for identical nucleotides. For the deletion construct (Del), the deleted region is marked with star character. (C) The enhancer activity of the rs953413 region is significantly decreased by mutations introduced into the predicted binding sites for HNF4α and FOXA factors (as illustrated in B) in luciferase assay. Luciferase construct Fragment 4 bearing the G allele of rs953413 was employed as template for mutation. For A and C, error bars, s.d. n = 6 technical replicates from two independent plasmid extractions and transfections with each transfection had three technical replicates. *p < 0.05; **p < 0.01 calculated by two-tailed Student’s t tests.

its proxies (r² > 0.8 in 1,000 Genomes phase 3) generally showed stronger association with EPA, DPA, and DHA levels compared with other SNPs (Figure S3). In addition, the rs953413 region showed tissue-specific H3K27ac signals with enrichment of H3K27ac binding only observed in HepG2, A549, and PANC-1 cells from all the available cell lines in the ENCODE project (Figure S4). The enrichment of H3K27ac over the rs953413 region was further validated with ChIP followed by quantitative real time (ChIP-qPCR) in liver cancer cell lines HepG2 and PLC/PRF/5 (Figure 1D). The enrichment of H3K27ac signal together with enrichment of TFs binding to the rs953413 region only in liver cells support the idea that the rs953413 region shows liver-specific enhancer feature (Figure S4). Interestingly, although not coming to genome-wide significant level, carriers of the G allele of rs953413 were observed to have higher expression of ELOVL2 (p = 1.4 × 10⁻⁶) in liver tissues from the GTEx project (Figure S5) (Battle et al., 2017). This observation coupled with the preferential binding of FOXA1 and FOXA2 to the G allele of rs953413 suggests that rs953413 may regulate ELOVL2 expression by modulating the enhancer activity of the rs953413 region in liver, which further affects the synthesis of LC-PUFAs.

The rs953413 Region Is Evolutionarily Conserved

To identify the causal TFs mediating the difference in enhancer activity in the rs953413 region, we first isolated the minimal enhancer (ME) element for this region. A series of truncation and deletion luciferase constructs were made with both genotypes of the original 529-bp fragments as templates and inserted upstream of the MP sequence as in Figure 1B. The ME element was mapped to a 184-bp fragment (Fragment 4) that showed strong enhancer activity while keeping the sequence at minimum (Figure 2A). This ME element includes a region of around 80-bp in length that is highly conserved in vertebrates and is essential for the observed enhancer activity as only luciferase constructs containing the whole conserved region (the original 529-bp fragment, Fragment 1, 4, and Truncate 3) showed strong enhancer activity. The sequences surrounding rs953413 are less conserved in vertebrates but are also essential for the enhancer activity as the luciferase construct containing only the evolutionarily conserved region (Truncate 2) had drastically decreased enhancer activity compared with luciferase construct containing both the conserved element and the rs953413 surrounding sequence (Truncate 3) (Figure 2A).

We then searched for TFs predicted to bind differently to the rs953413 region with the ME element as template and using the TRAP tool with position weight matrices (PWMs) from both the JASPAR database and literature as references (Mathelier et al., 2014; Thomas-Chollier et al., 2011). In accordance with the observed allelic imbalance in chromatin binding for FOXA1 and FOXA2 in ChIP-seq, we identified multiple highly conserved FOXA motifs together with two conserved HNF4α motifs in the ME element (Figure 2B). The predicted motifs are located in evolutionarily conserved sequences denoted by the basewise conservation in 100 vertebrates from the ENCODE project (Figure 2B). The variant rs953413 is predicted to be located in two closely located motifs bound by FOXA and HNF4α indicative of cooperative binding by these TFs (Jolma et al., 2015).

We next examined the effect of the predicted TF binding sites on the enhancer activity of the rs953413 region by site-directed mutagenesis. Fragment 4 bearing the G allele of rs953413 and containing the whole ME element was employed as the template for construction of mutants (Figure 2B). We observed that introducing mutations at any of the predicted TF binding sites significantly decreased the enhancer activity of the rs953413 region (Figure 2C). The enhancer activity was completely depleted when mutations were introduced to the predicted binding sites in highly conserved region (constructs FX-mut1 and HF-mut1). Notably, introducing mutations to the predicted binding sites overlapping with rs953413 (constructs FX-mut4, HF-mut2, and Del) also severely decreased the enhancer activity, suggesting that rs953413 is functional in determining the enhancer activity of this region (Figures 2B and 2C).
FOXA1 and FOXA2 Regulates ELOVL2 Expression by Binding to the rs953413 Region

We next examined if both FOXA1 and FOXA2 are directly involved in the regulation of the enhancer activity of the rs953413 region. In accordance with our motif prediction, the minimal region that responds to both FOXA1 and FOXA2 induction is mapped to the luciferase construct containing the whole ME element (Fragment 4) from a series of deletion constructs in luciferase assay (Figure S6). Both alleles of rs953413 were highly induced by both FOXA1 and FOXA2 overexpression (Figure 3A). The difference in enhancer activity between alleles of rs953413 was significantly compromised by FOXA overexpression probably due to presence of multiple conserved FOXA binding sites in this region. The responses to both FOXA1 and FOXA2 overexpression were significantly decreased by introducing mutations to the predicted FOXA binding sites in luciferase construct Fragment 4 bearing the G allele of rs953413. Introducing mutations to the predicted HNF4α binding sites also made this region less responsive to both FOXA1 and FOXA2 overexpression with significantly decreased enhancer activities compared with the wild type (Figure 3B). Especially, the response to FOXA1/FOXA2 overexpression was completely depleted in luciferase construct HF-mut1 with one of the predicted HNF4α binding sites being mutated, suggesting that binding of both FOXA1/FOXA2 and HNF4α to the rs953413 region is essential for the enhancer activity.

We next assessed the in vivo binding of both FOXA1 and FOXA2 to the rs953413 region through ChIP-qPCR. In accordance with the enrichment of ChIP-seq signals for both FOXA1 and FOXA2 in this region in HepG2 cells from the ENCODE project, the rs953413 region was observed to be highly enriched with FOXA1 and FOXA2 binding in parallel with the positive control region HNF1α-pro after normalization with the negative control region GDCHR12 in both HepG2 and PLC/PRF/5 cells (Figure 3C). To address whether FOXA1/FOXA2 showed allele-specific DNA binding at rs953413 in vivo, we conducted ChIP followed by allele-specific qPCR (AS-qPCR). The allele-specific amplification of each allele was achieved with primers designed following mismatch amplification mutation assay (MAMA) and validated with genomic DNA from samples bearing different genotypes at rs953413 as templates (Figure S7) (Cha et al., 1992; Li et al., 2004). In accordance with the observed preference for the G allele in ChIP-seq experiments, both FOXA1 and FOXA2 were identified to be preferentially bound to the G allele of rs953413 in ChIP followed by AS-qPCR in both HepG2 and PLC/PRF/5 cells (Figure 3D). This allelic imbalance in chromatin binding for both FOXA1 and FOXA2 at the rs953413 region was further confirmed with Sanger sequencing analysis of the rs953413-containing region in chromatin fragments immunoprecipitated with antibody against both FOXA1 and FOXA2 compared with that in the input genomic DNA as control (Figure 3E). Additionally, the G allele of rs953413 was significantly more enriched with H3K27ac signal compared with the A allele in ChIP samples from Figure 1D in both HepG2 and PLC/PRF/5 cells, suggesting more of FOXA binding in this region leading to stronger enhancer activity (Figure 3F).

To determine if FOXA1 and FOXA2 are directly involved in regulation of ELOVL2 expression, we next performed lentiviral-mediated FOXA1 knockdown through short hairpin RNA (shRNA) and artificial microRNA (amiRNA) in HepG2 and PLC/PRF/5 cells (Liang et al., 2012). Knockdown of FOXA1 significantly decreased the expression of ELOVL2 in both cells after normalization to reference genes RSP18, ACTB, and GAPDH, respectively (Figures 3G and S8). The expression of FOXA2 is significantly increased by FOXA1 knockdown in HepG2 cells, whereas it is observed to be significantly downregulated by FOXA1 knockdown in PLC/PRF/5 cells, indicative of cell heterogeneity between these two cells. The A allele of exonic SNP rs2295601 of ELOVL2 is on the same haplotype as the A allele of rs953413 evaluated with the data from the 1,000 Genomes Project (Machiela and Chanock, 2015). The efficiency and specificity of AS-qPCR assay targeting rs2295601 was validated with genomic DNA from the same set of samples for rs953413 AS-qPCR as templates (Figure S7). We have evidence that FOXA1/FOXA2 favor binding to the G allele of rs953413 (Figures 3D and 3E). We therefore evaluated whether this allele-specific binding caused allelic imbalance in ELOVL2 expression with rs2295601 AS-qPCR in HepG2 cells, which is heterozygous at this location, whereas PLC/PRF/5 was identified to be homozygous with the G allele. This analysis identified that the G allele of rs2295601 in linkage with the G allele of rs953413 was selectively transcribed in HepG2 cells. Knockdown of FOXA1 significantly diminished the allelic imbalance in ELOVL2 expression (Figure 3H).

HNF4α Regulates ELOVL2 Expression through Interacting with the rs953413 Region

We next assessed whether HNF4α is also directly involved in ELOVL2 regulation through interacting with the rs953413 region. We observed that luciferase construct (Fragment 5) encompassing both of the predicted HNF4α binding sites was highly induced by HNF4α overexpression (Figure 4A). To refine the
Figure 3. FOXA Factors Directly Bind to the ME Element and Confer Allelic Imbalance in ELOVL2 Expression Induced by rs953413

(A) The enhancer activity of the rs953413 region is highly induced by both FOXA1 and FOXA2 overexpression in luciferase assay. (B) The response to either FOXA1 or FOXA2 overexpression is significantly compromised by the mutations introduced into the predicted binding sites for HNF4α and FOXA factors in luciferase assay. In A and B, error bars, s.d. n = 6 technical replicates from two independent plasmid extractions and transfections with each transfection had three technical replicates. (C) Both FOXA1 and FOXA2 are highly enriched in the rs953413 region verified by ChIP-qPCR in both HepG2 and PLC/PRF/5 cells. Error bars, s.d. (D and E) FOXA1 and FOXA2 favor binding to the G allele of rs953413 as determined by ChIP followed by AS-qPCR (D) and ChIP followed by PCR amplification and Sanger sequencing (E) in both HepG2 and PLC/PRF/5 cells. The input for ChIP was used as the control. *p < 0.05 and **p < 0.01 calculated by two-tailed Student’s t tests. Error bars, s.d. In C and D, n = 4 for HepG2 cells and n = 3 for PLC/PRF/5 cells. (F) The G allele of rs953413 is also significantly more enriched with H3K27ac signal compared with the A allele determined by ChIP followed by AS-qPCR. n = 3. Error bars, s.d. **p < 0.01 calculated by two-tailed Student’s t tests. (G) The expression of ELOVL2 is significantly downregulated by FOXA1 knockdown in HepG2 and PLC/PRF/5 cells. (H) The allelic imbalance in ELOVL2 expression is significantly decreased by FOXA1 knockdown in HepG2 cells. The raw ratios are shown here without correcting for the CNV. In G and H, **p < 0.01 and ns, not significant, calculated by two-tailed Student’s t tests. Error bars, s.d. n = 8 technical replicates.

See also Figures S6–S8.
Figure 4. HNF4α regulates ELOVL2 Expression by Directly Binding to the ME Element and Confers Allelic Imbalance in ELOVL2 Expression by rs953413

(A) Both of the predicted HNF4α binding sites in the ME element are essential for the maximal induction by HNF4α in luciferase assay.

(B) Disruption either of the predicted HNF4α-binding sites significantly decreased the enhancer activity induced by HNF4α in luciferase assay. In A and B, error bars, s.d. n = 6 technical replicates from two independent plasmid extractions and transfections with each transfection had three technical replicates.

(C) The rs953413 region is highly enriched with HNF4α binding compared with GDCHR12 in ChIP-qPCR. Error bars, s.d. (D and E) HNF4α favors binding to the G allele of rs953413 relative to the A allele as determined by ChIP followed by AS-qPCR (D) and ChIP followed by PCR amplification and Sanger sequencing (E) in both HepG2 and PLC/PRF/5 cells. The input for ChIP was used as the control. **p < 0.01 calculated by two-tailed Student’s t tests. Error bars, s.d. In C and D, n = 4 for HepG2 cells and n = 3 for PLC/PRF/5 cells.

(F) The expression of ELOVL2 is significantly decreased by HNF4α knockdown in both HepG2 and PLC/PRF/5 cells.

(G) The allelic imbalance in ELOVL2 expression is significantly decreased by HNF4α knockdown in HepG2 cells. The raw ratios are shown here without correcting for the CNV. In F and G, error bars, s.d. n = 8 technical replicates.

See also Figures S7 and S9.
**HNF4α-Responsive Regions, Truncation and Deletion Luciferase Constructs Were Further Constructed and Subjected to HNF4α Overexpression in Luciferase Assay.** In accordance with our prediction, only constructs containing the predicted HNF4α-binding sites (Fragment 5, Truncate 4 and 5) were induced by HNF4α overexpression. To map the sequences needed for HNF4α induction, we introduced mutations to either of the predicted HNF4α sites with Fragment 5 as template that led to drastically diminished HNF4α induction (Figure 4B). Intriguingly, the Del construct (as illustrated in Figure 2B) with the predicted binding sites for HNF4α and FOXA factors surrounding rs953413 being removed completely abolished the HNF4α induction, suggesting rs953413 is directly located in one functional HNF4α-binding site.

We next performed ChIP-qPCR assays with antibody against HNF4α in both HepG2 and PLC/PRF/5 cells. In accordance with the data from the ENCODE project, we confirmed HNF4α chromatin binding at the rs953413 region and at the positive HNF1α-pro region after normalization with GCDFR12 (Figure 4C). To address whether HNF4α showed allele-specific DNA binding at rs953413 in vivo, we conducted ChIP followed by rs953413 AS-qPCR. This analysis showed that HNF4α was preferentially bound to the G allele of rs953413 in both HepG2 and PLC/PRF/5 cells (Figure 4D). Consistent with this finding, Sanger sequencing analysis of the rs953413-containing region showed that the G allele was enriched in chromatin fragments immunoprecipitated with antibody against HNF4α compared with the input genomic DNA as control in both cells (Figure 4E). We next performed lentiviral-mediated HNF4α knockdown through shRNA and amiRNA to test its effect on ELOVL2 expression (Liang et al., 2012). Knockdown of HNF4α resulted in significantly decreased expression of ELOVL2 after normalization to reference genes RSP18, ACTB, and GAPDH, respectively (Figures 4F and S9). We then performed rs2295601 AS-qPCR to evaluate if the allelic imbalance in ELOVL2 expression was affected by HNF4α knockdown. After HNF4α knockdown, the allelic imbalance in ELOVL2 expression was also observed to be significantly decreasing (Figure 4G).

**Cooperation Between HNF4α and FOXA Factors Determine the Enhancer Activity of the rs953413 Region**

As both HNF4α and FOXA factors are bound to the rs953413 region and essential for its enhancer activity, we hypothesized that HNF4α and FOXA factors may cooperate with each other in a complex to determine the enhancer activity of the rs953413 region, which further regulates ELOVL2 expression. To test this hypothesis, we first carried out luciferase assay overexpressing both HNF4α and FOXA factors together with luciferase construct Fragment 4 containing the whole ME element. Compared with overexpression of HNF4α alone or either FOXA1 or FOXA2 alone, the enhancer activities of both alleles of rs953413 were significantly increased by overexpression of HNF4α together with either FOXA1 or FOXA2, indicative of cooperation between these TFs in determining the enhancer activity of the rs953413 region (Figures S5A and S10).

To determine the effect of the cooperation between HNF4α and FOXA factors on ELOVL2 expression, we generated HepG2 and PLC/PRF/5 cells with both HNF4α and FOXA1 being knocked down. This double knockdown was achieved with either sequential or simultaneous transduction of lentivirus for HNF4α and FOXA1 knockdown. The double knockdown cells produced with simultaneous knockdown were named FOXA1 + HNF4α KD. FOXA1-KD + HNF4α-KD denotes cells first transduced with lentivirus for FOXA1 knockdown followed by HNF4α knockdown and vice versa for HNF4α-KD + FOXA1-KD. To be comparable, the control cells and cells with knockdown of only FOXA1 or HNF4α were also subjected to two rounds of lentiviral transduction. Compared with knockdown of only HNF4α or FOXA1, this double knockdown strategy led to significant downregulation of not only HNF4α and FOXA1 but also FOXA2 in both cells after normalization to reference genes RSP18, ACTB, and GAPDH, respectively (Figures 5B–5D and S11). It is noteworthy that the degree of knockdown for all the three TFs is much stronger compared with single knockdown in HepG2 cells (Figure 5C). As HNF4α and FOXA1/FOXA2 are all members of the hepatocyte nuclear factor family, the cross-regulation of one another and the synergy in gene regulation has been well studied (Lau et al., 2018). We investigated ChIP-seq signals for histone modifications (H3K4me3 and H3K27ac) in HepG2 cells indicating promoters and enhancers over the gene bodies of all the three TFs. We found that ChIP-seq signals for all the three TFs are enriched over the active chromatin regions of each TF’s gene body indicative of coregulation of all the three TFs by themselves (Figure S12). Coupling to the knockdown of all the three TFs, the expression of ELOVL2 was significantly downregulated compared with knockdown of only HNF4α or FOXA1 in both HepG2 and PLC/PRF/5 cells (Figures 5B and S11). In addition, contrary to the observed decrease in allelic imbalance in ELOVL2 expression after knockdown of only HNF4α or FOXA1 in HepG2 cells, the triple knockdown led to a significant increase in the allelic imbalance in ELOVL2 expression (Figure 5E). Interestingly, the degree of allelic imbalance in ELOVL2...
Figure 5. Cooperation between HNF4α and FOXA Factors Determines the Enhancer Activity of the rs953413 Region and ELOVL2 Expression

(A) The enhancer activity of the rs953413 region was significantly increased by simultaneous overexpression of HNF4α and FOXA1 compared with overexpression of only HNF4α or FOXA1 in luciferase assay. Error bars, s.d. n = 6 technical replicates from two independent plasmid extractions and transfections with each transfection had three technical replicates.

(B–D) Double knockdown of both HNF4α and FOXA1 led to a significant decrease in ELOVL2 expression compared with knockdown only HNF4α or FOXA1 (B). This double knockdown also significantly downregulated FOXA2 expression in both HepG2 cells (C) and PLC/PRF/5 cells (D).

(E and F) The allelic imbalance in ELOVL2 expression shown in E is gradually increased by the gradual decreases in expression of HNF4α and FOXA1/FOXA2 in different double-knockdown HepG2 cells as shown in F. In B–F, **p < 0.01 calculated by two-tailed Student’s t tests. Error bars, s.d. n = 4 technical replicates.

See also Figures S10 and S11.
Figure 6. CRISP/Cas9-mediated Mutation of the ME Element Impairs ELOVL2 Expression in an Allele-Specific Manner

(A) Schematic illustrating CRISP/Cas9-mediated mutation of the ME element by double gRNA. The ME element is highlighted in blue color. The binding sites for the primer pair used to validate mutations are indicated.
The rs953413 Region Works as a Key Enhancer Region for ELOVL2 Regulation

To provide more direct evidence that the rs953413 region is involved in ELOVL2 regulation, we first conducted CRISPR/Cas9-mediated mutagenesis of this region in HepG2 cells. Two guide RNAs were designed to cut the flanking sequences on both sides of the ME element to delete the whole rs953413 region (Figure 6A). After selecting multiple colonies, we did not get single colonies homozygous with each allele of the ME element being deleted due to the existence of CNV in this region. However, we observed that the expression of ELOVL2 together with the allelic imbalance in ELOVL2 expression was significantly different among different single colonies with different mutation profiles in the rs953413 region (Figure 6B). To determine the causal relationship between the mutations introduced into the rs953413 region with ELOVL2 expression, we selected nineteen single colonies with different mutation profiles for further characterization. Sanger sequencing on direct PCR product amplified from the rs953413 region was carried out from both sides to determine the mutation pattern for each colony together with multiple (from 5 to 26) TA clones of PCR product to determine the genotype of each colony. From all the selected colonies, we successfully identified the genotypes of twelve colonies, with the others either having multiple alleles detected or missing the A allele of rs953413 detected.

Our previous findings suggest that the predicted binding sites for FOXA factors are essential for the enhancer activity of the rs953413 region as mutation construct FX-mut1 completely lost the enhancer activity and its response to both FOXA1 and FOXA2 induction in luciferase assay (Figure 3B). The twelve colonies can generally be categorized into two groups based on the location of the mutations introduced. The alleles of the control group only have mutations flanking the ME element and the mut group have mutations to the ME element that also disrupted the predicted binding sites for FOXA factors as in mutation construct FX-mut1 (Figure 6C). Four colonies were categorized as control (Figure S13) and eight colonies fell into the category of mut with five colonies having critical mutations in one copy of the ME element bearing the G allele of rs953413 (G1; Figure S14), one colony had mutations in both copies of ME element bearing the G allele of rs953413 (G2; Figure S15A) and two colonies had all the alleles of the ME elements mutated (all; Figure S15B). In accordance with our previous results, the colonies with mutations in the predicted FOXA-binding sites (mut) had significantly decreased expression of ELOVL2 compared with the control group (control) after normalization with reference genes RSP18, ACTB, and GAPDH, respectively (Figures 6D and S16). Accordingly, the allelic imbalance in ELOVL2 expression detected by rs2295601 AS-gPCR was significantly decreased in both group G1 and G2 compared with the control group in a dosage-dependent manner (Figure 6E). In addition, the two colonies grouped with all the copies of the
ME elements being mutated (all) had relatively more conserved FOXA binding sites in the ME elements bearing the G allele of rs953413 compared with that of the A allele of rs953413 that led to significantly increased allelic imbalance in ELOVL2 expression (Figure S17). This suggested that the ME element encompassing rs953413 is a key regulatory region for ELOVL2 expression.

We next coupled each AS-gRNA with wild-type Cas9 to directly introduce mutations to rs953413 to determine its effect on ELOVL2 expression in HepG2 cells. The allelic imbalance in mutating rs953413 by each AS-gRNA was determined with rs953413 AS-qPCR, which can specifically quantify the relative amount of mutations introduced into each allele of rs953413. In accordance with our previous observations, the A allele of rs953413 was preferentially mutated by A-gRNA coupled with wild-type Cas9, whereas the G allele of rs953413 was preferentially mutated by G-gRNA compared with cells transduced with lentiviral transduction vector expressing Cas9 (Figure 6J). The expression of ELOVL2 was observed to be significantly downregulated by mutations introduced to rs953413 by each AS-gRNA after normalization with reference genes RSP18, ACTB, and GAPDH, respectively (Figures 6F and S18). However, the rs2295601 AS-qPCR clearly showed that both AS-gRNA displayed high allelic preference in affecting ELOVL2 expression (Figure 6F). When coupled with Cas9-KRAB, each AS-gRNA preferentially suppressed the transcription of ELOVL2 located on the same chromosome with target allele of rs953413. While coupling with Cas9-VP64, the ELOVL2 transcript in linkage with respective allele of rs953413 was selectively activated by AS-gRNA.

We here demonstrate that rs953413 mediates the cooperative binding of HNF4α and FOXA1/FOXA2 to the evolutionarily conserved enhancer region, which acts as a key cis-regulatory element for ELOVL2 expression. The G allele of rs953413 is preferentially bound by the complex formed by these TFs and increases the expression of ELOVL2 further to increase the amount of LC-PUFAs synthesized (Figure 6M).

As members of the hepatocyte nuclear factor family, HNF4α and FOXA1/FOXA2 are all essential TFs for normal liver function and their expression profile also showed high tissue specificities with a preference for the liver, pancreas, and kidney (Lau et al., 2018). In accordance with the enhancer activity tightly regulated by the cooperation between FOXA1/FOXA2 and HNF4α, the rs953413 region showed tissue-specific enhancer features with both active chromatin marker H3K27ac and different TFs enriched mainly in liver HepG2 cells (Figure S4). As liver is the most important organ for LC-PUFAs synthesis, the rs953413 region is supposed to be a key enhancer region for ELOVL2 regulation in the liver, which further determines the systemic LC-PUFAs profiles. In accordance with the G allele of rs953413 upregulating ELOVL2 expression in our study, SNPs in LD with the G allele of rs953413 were generally associated with a more efficient conversion of DHA from its precursors in GWAS (Draisma et al., 2015; Illig et al., 2009; Lemaitre et al., 2011; Li et al., 2018; Suhre et al., 2011). The expression of ELOVL2 in other tissues might be regulated by different mechanisms other than the rs953413 region illustrated by the observation that the A allele of rs953413 was associated with higher expression of ELOVL2 in transformed fibroblasts (p = 1.5 × 10⁻⁶) from the GTEx project.
Additionally, it should be noted that an independent association between SNPs indexed by rs2281591 in the ELOVL2 locus with DPA levels was observed in conditional analyses in both European- and Chinese-ancestry populations (Hu et al., 2016; Lemaitre et al., 2011). As these SNPs are not in LD with rs953413, this independent association signal suggests that there are other functional variants in this locus involved in ELOVL2 regulation.

Previous studies have shown that ω-3 PUFAs, particularly DHA, may influence human health by exerting beneficial effects on many diseases such as cardiovascular disease, diabetes, cancer, depression, nonalcoholic fatty liver disease (NAFLD), and rheumatoid arthritis (Jump et al., 2018; Lopez-Vicario et al., 2014; Shahidi and Ambigaipalan, 2018). Recently, rs2236212, an intronic SNP of ELOVL2, was shown to be associated with NAFLD in obese subjects (Zusi et al., 2019). Patients and animal models of nonalcoholic steatohepatitis (NASH), a severe form of NAFLD, showed marked decrease in hepatic ω-3 PUFAs levels, which may play a role in the development and progression of NASH (Jump et al., 2018; Lopez-Vicario et al., 2014). Dietary intervention with ω-3 PUFAs DHA alone or EPA/DHA have shown indications of improvement in biomarkers related to NASH. The identified TFs in this study, including FOXA1/FOXA2 and HNF4α, are tightly involved in NAFLD progression and are significantly downregulated in NAFLD (Lake et al., 2016; Moya et al., 2012; Wang et al., 2017; Weiss et al., 2017). This implies that downregulation of ELOVL2 together with its upstream regulators FOXA1/FOXA2 and HNF4α impairs hepatic DHA synthesis, which may play a role in the pathogenesis of NAFLD.

As a key enzyme in the in vivo synthesis of DHA, dysregulation of ELOVL2 may also be involved in impairment of the systemic inflammatory process and diabetes progression (Bellini et al., 2018; Cruciani-Guglielmacci et al., 2017; Talamonti et al., 2017; Tikhonenko et al., 2010). However, further studies are needed to investigate the key TFs regulating ELOVL2 in other cell types. It is interesting to note that HNF4α, FOXA1, and FOXA2 have been extensively studied and appears to be involved in the progression of diabetes, and variations nearby HNF4α and FOXA2 have been reported to be associated with diabetes related traits in GWAS (Lau et al., 2018; MacArthur et al., 2017). ELOVL2 may be an important downstream target in diabetes caused by dysregulation of these TFs.

In conclusion, we show that rs953413 affects LC-PUFAs levels by altering ELOVL2 expression through FOXA1/FOXA2 and HNF4α cooperation. The results provide important mechanistic insights to the transcriptional machinery regulating ELOVL2 in the liver and thereby circulating levels of PUFAs. Further studies are needed to elucidate the role of this pathway in diseases, liver diseases in particular.

Limitations of the Study
Due to lack of detailed genotype and phenotype data at individual level in reported GWAS in this locus, the exact effect of rs953413 and other variations conditional on rs953413 on the reported phenotypes cannot be determined. As the exonic SNP rs2295601 is homozygous in PLC/PRF/5 cells, the changes in allelic imbalance in ELOVL2 expression upon modulating the rs953413 locus cannot be determined in this cell line.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100808.

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AUTHOR CONTRIBUTIONS
C.W. conceived and oversaw this study. G.P. performed the experiments. G.P. and C.W. analyzed the data and wrote the manuscript with input from M.C., B.C., S.S., and C.K.. All authors approved the manuscript.
DECLARATION OF INTERESTS
B.C., S.S., and C.K. are employees of AstraZeneca and may own stock or stock options. The remaining authors declare no competing interests.

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Supplemental Information

rs953413 Regulates Polyunsaturated Fatty Acid Metabolism

by Modulating ELOVL2 Expression

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Figure S1. The reported tag SNPs in the ELOVL2 locus associated with LC-PUFAs derived metabolites in GWAS are in high LD. Related to Figure 1. The tag SNPs are from the GWAS listed in Table S1. The color in each well denotes LOD value while the number denotes $r^2$ value between pair of SNPs in European population (CEU in 1000 Genomes Phase 3).
Figure S2. Luciferase assay on the rs3798713 region. Related to Figure 1. Both rs3798713 and rs17675073 are AS-SNPs in HepG2 cells that are in proximity. To evaluate the effect of each SNP, luciferase constructs containing different genotypes were constructed by direct PCR and site-directed mutagen with primers listed in Table S4. Error bars, s.d. $n=6$ from two independent plasmid extractions and transfections with each transfection had three technical replicates. $P$ values were calculated using two-tailed Student’s $t$ tests.
Figure S3. rs953413 and its proxies showed more statistical powder in associating with levels of ω-3 fatty acids in plasma phospholipid. Related to Figure 1. All the significant SNPs in the ELOVL2 locus associated with EPA (A), DPA (B) and DHA (C) in the meta-analysis carried out in the CHARGE Consortium were plotted (Lemaitre et al., 2011). The plots were generated with rs953413 as the reference and LD is indicated by color scale in relation to rs953413 ($r^2$ values calculated in EUR in 1000 Genomes Phase 3).
Figure S4. rs953413 is located in a liver-specific enhancer region. Related to Figure 1. A 5-kb window with rs953413 in the center is displayed here. The H3K27ac signals in HepG2 and other cell lines from the ENCODE project are shown for this region (The Encode Project Consortium, 2012). The exact location of rs953413 is highlighted by a vertical black line. The rs953413-containing region is also bound by many TFs in the liver which is identified by ChIP-seq experiments with antibodies against different TFs from the ENCODE project and is shown at the top.
| Tissue                                                                 | Samples | NES  | p-value     | m-value | Single-tissue eQTL NES (with 95% CI) |
|-----------------------------------------------------------------------|---------|------|-------------|---------|-----------------------------------|
| Colon - Sigmoid                                                       | 203     | 0.266| 3.2e-5      | 1.00    |                                   |
| Cells - Transformed fibroblasts                                       | 300     | 0.258| 1.5e-6      | 1.00    |                                   |
| Esophagus - Gastroesophageal Junction                                | 213     | 0.226| 2.1e-3      | 0.998   |                                   |
| Brain - Anterior cingulate cortex (BA24)                            | 109     | 0.223| 1.1e-5      | 1.00    |                                   |
| Heart - Left Ventricle                                               | 272     | 0.206| 3.2e-4      | 1.00    |                                   |
| Cells - EBV-transformed lymphocytes                                  | 117     | 0.204| 0.08        | 0.911   |                                   |
| Brain - Caudate (basal ganglia)                                      | 144     | 0.189| 4.0e-4      | 1.00    |                                   |
| Brain - Cerebellum                                                   | 154     | 0.163| 3.2e-5      | 1.00    |                                   |
| Brain - Cortex                                                       | 136     | 0.180| 3.8e-5      | 1.00    |                                   |
| Esophagus - Muscularis                                               | 335     | 0.164| 1.2e-3      | 1.00    |                                   |
| Brain - Amygdala                                                     | 88      | 0.163| 0.02        | 0.950   |                                   |
| Pancreas                                                             | 220     | 0.160| 0.02        | 0.956   |                                   |
| Brain - Frontal Cortex (BA3)                                         | 118     | 0.157| 1.0e-3      | 1.00    |                                   |
| Ovary                                                                | 122     | 0.154| 0.2         | 0.792   |                                   |
| Brain - Nucleus accumbens (basal ganglia)                           | 130     | 0.154| 4.7e-3      | 0.991   |                                   |
| Prostate                                                             | 132     | 0.137| 0.07        | 0.942   |                                   |
| Thyroid                                                              | 399     | 0.132| 0.01        | 0.965   |                                   |
| Testis                                                                | 225     | 0.123| 0.05        | 0.944   |                                   |
| Muscle - Skeletal                                                    | 491     | 0.121| 0.02        | 0.961   |                                   |
| Brain - Cerebellar Hemisphere                                        | 125     | 0.114| 0.01        | 0.947   |                                   |
| Brain - Hippocampus                                                  | 111     | 0.113| 0.06        | 0.844   |                                   |
| Minor Salivary Gland                                                 | 85      | 0.110| 0.4         | 0.682   |                                   |
| Nerve - Tibial                                                       | 361     | 0.100| 0.03        | 0.940   |                                   |
| Brain - Putamen (basal ganglia)                                      | 111     | 0.095| 0.1         | 0.832   |                                   |
| Adrenal Gland                                                        | 175     | 0.0865| 0.4         | 0.611   |                                   |
| Brain - Spinal cord (cervical c-1)                                   | 63      | 0.0815| 0.3         | 0.764   |                                   |
| Esophagus - Mucosa                                                   | 358     | 0.0757| 0.1         | 0.672   |                                   |
| Small Intestine - Terminal ileum                                    | 122     | 0.0744| 0.4         | 0.752   |                                   |
| Adipose - Subcutaneous                                               | 385     | 0.0699| 0.1         | 0.529   |                                   |
| Artery - Coronary                                                    | 152     | 0.0694| 0.3         | 0.602   |                                   |
| Skin - Not Sun Exposed (Suprapubic)                                  | 335     | 0.0588| 0.4         | 0.431   |                                   |
| Colon - Transverse                                                   | 245     | 0.0501| 0.3         | 0.274   |                                   |
| Brain - Hypothalamus                                                 | 108     | 0.0463| 0.6         | 0.447   |                                   |
| Skin - Sun Exposed (Lower leg)                                       | 414     | 0.0265| 0.6         | 0.145   |                                   |
| Brain - Substantia nigra                                             | 80      | 0.0252| 0.8         | 0.517   |                                   |
| Uterus                                                               | 101     | 0.0250| 0.9         | 0.682   |                                   |
| Artery - Tibial                                                      | 388     | 0.0146| 0.7         | 0.002000|                                   |
| Stomach                                                              | 237     | 0.0143| 0.8         | 0.0830  |                                   |
| Heart - Atrial Appendage                                            | 264     | 0.00896| 0.9        | 0.135   |                                   |
| Pituitary                                                            | 157     | 0.00159| 1          | 0.162   |                                   |
| Whole Blood                                                          | 369     | -     | -           | -       |                                   |
| Spleen                                                               | 146     | -     | -           | -       |                                   |
| Adipose - Visceral (Omentum)                                         | 313     | -0.00256| 1         | 0.126   |                                   |
| Breast - Mammary Tissue                                             | 251     | -0.0178| 0.7        | 0.00500 |                                   |
| Lung                                                                 | 383     | -0.0270| 0.6        | 0.0100  |                                   |
| Artery - Aorta                                                       | 267     | -0.0371| 0.5        | 0.00800 |                                   |
| Vagina                                                               | 106     | -0.0636| 0.5        | 0.143   |                                   |
| Liver                                                                | 153     | -0.212| 1.4e-4     | 0.00    |                                   |
Figure S5. The eQTL analysis of rs953413 on ELOVL2 expression in multiple tissues from the GTEx portal. Related to Figure 1. The figure was directly downloaded from the GTEx portal with minor modifications (Battle et al., 2017). NES, normalized effect size. In the liver tissue, the A allele of rs953413 is associated with decreased expression of ELOVL2 with NES of -0.212.

![Figure S5](image)

Figure S6. The whole ME element is essential for the enhancer activity of the rs953413 region induced by FOXA1 and FOXA2. Related to Figure 3. Only luciferase construct contains the whole ME element (Fragment 4) could be induced by FOXA1 and FOXA2 overexpression. Error bars, s.d. n = 6 from two independent plasmid extractions and transfections with each transfection had three technical replicates.

![Figure S6](image)
Figure S7. Validation of AS-qPCR primers for rs953413 and rs2295601 with genomic DNA from different samples as templates. Related to Figure 3-6. The $y$ axis shows the $C_T$ value differences in qPCR with different genomic DNA as templates for both rs953413[G] versus rs953413[A] (dark grey bar) and rs2295601[G] versus rs2295601[A] (light grey bar). The exact Δ$C_T$ values are displayed for each experiment. The determined genotypes for both rs953413 and rs2295601 are the same in each sample and are listed on $x$ axis. Due to existence of two copies of the G allele in HepG2 cells, the Δ$C_T$ values in HepG2 cells for both SNPs are different from the normal heterozygous sample.
Figure S8. The expression of ELOVL2 is significantly decreased by FOXA1 knockdown. Related to Figure 3. The relative expression of each target gene was normalized with the expression of ACTB (A) and GAPDH (B). *P < 0.05; **P < 0.01 and ns, not significant calculated by two-tailed Student’s t tests. Error bars, s.d. n = 8 technical replicates from two independent experiments with each experiment had four replicates.

Figure S9. The expression of ELOVL2 is significantly decreased by HNF4α knockdown. Related to Figure 4. The relative expression of each detected gene was normalized with the expression of ACTB (A) and GAPDH (B). *P < 0.05; **P < 0.01 and ns, not significant calculated by two-tailed Student’s t tests. Error bars, s.d. n = 8 technical replicates from two independent experiments with each experiment had four replicates.
Figure S10. Cooperation between HNF4α and FOXA2 significantly increased the enhancer activity of the rs953413 region in luciferase assay. Related to Figure 5. The enhancer activity of the rs953413 region is significantly increased by simultaneous overexpression of HNF4α and FOXA2 compared with overexpression only HNF4α or FOXA2 in luciferase assay. Error bars, s.d. $n = 6$ technical replicates from two independent plasmid extractions and transfections with each transfection had three technical replicates.
Figure S11. Double knockdown of both HNF4α and FOXA1 leads to a significant decrease in ELOVL2 expression compared with knockdown only HNF4α or FOXA1. Related to Figure 5. The relative expression of each gene is normalized to the expression of ACTB (A, B) and GAPDH (C, D) in both HepG2 and PLC/PRF/5 cells. The double knockdown cell lines were generated by either sequential or simultaneous lentivirus transduction. FOXA1-KD+HNF4α-KD denotes the cell line first transduced with the virus for FOXA1 knockdown and then transduced the virus for HNF4α knockdown and vice versa for HNF4α-KD+FOXA1-KD. FOXA1+HNF4α KD denotes the cell line with simultaneously transduced virus for both HNF4α and FOXA1 knockdown. **P < 0.01 calculated by two-tailed Student’s t tests. Error bars, s.d. n = 4 technical replicates.
Figure S12. ChIP-seq signals of HNF4α, FOXA1 and FOXA2 from the ENCODE project are all enriched over the gene body of HNF4α, FOXA1 and FOXA2, respectively in HepG2 cells. Related to Figure 5. The ChIP-seq signals of HNF4α, FOXA1 and FOXA2 together with H3K27ac and H3K4me3 signals in HepG2 cells are displayed over the gene body of HNF4α (A), FOXA1 (B) and FOXA2 (C).

Figure S13. Individual clones bearing no mutations to the ME element identified by Sanger sequencing. Related to Figure 6. For each clone, genomic DNA was PCR-amplified and subjected to both direct PCR product sequencing and TA cloning sequencing. The ME element is highlighted in blue. The arrow denotes the expected Cas9 cut sites. Base-pair changes are shown in red and deletions are represented by dashes.
Figure S14. Individual clones with mutations introduced to one copy of the ME element bearing the G allele of rs953413 identified by Sanger sequencing. Related to Figure 6. For each clone, genomic DNA was PCR-amplified and subjected to both direct PCR product sequencing and TA cloning sequencing. The ME element is highlighted in blue. The arrow denotes the expected Cas9 cut sites. Base-pair changes are shown in red and deletions are represented by dashes.
Figure S15. Individual clones with mutations introduced to multiple copies of the ME element identified by Sanger sequencing. Related to Figure 6. (A) clone 9 introduced mutations to both copies of the ME element bearing the G allele of rs953413. (B) clone 3 and clone 20 are verified to have mutations to all copies of the ME element. For each clone, genomic DNA was PCR-amplified and subjected to both direct PCR product sequencing and TA cloning sequencing. The ME element is highlighted in blue. The arrow denotes the expected Cas9 cut sites. Base-pair changes are shown in red and deletions are represented by dashes.
Figure S16. The clones bearing mutations in the ME element (mut) had impaired ELOVL2 expression compared with clones without mutations introduced in the ME element (control). Related to Figure 6. The expression of ELOVL2 is either normalized to the expression of ACTB (A) or GAPDH (B). Each clone has three technical replicates. P values were evaluated using two-tailed Student’s t tests. WT denotes ELOVL2 expression from normal HepG2 cells (n=3).

Figure S17. Increased allelic imbalance in ELOVL2 expression in clone 3 and clone 20. Related to Figure 6. (A) In both clone 3 and clone 20, the ME element of each allele is mutated and the ME elements bearing the G allele of rs953413 have a more conserved FOXA motif compared to the A allele (see also Figure S15B). WT represents the wild type sequence while allele G and allele A represent the mutated ME sequences in linkage with rs953413[G] and rs953413[A], respectively. The mutated key motif sequences are displayed in lower-case letters and highlighted in red. (B) The increased allelic imbalance in ELOVL2...
expression is detected by rs2295601 AS-qPCR. A significant increase in allelic imbalance of ELOVL2 expression is observed in clone 3 and clone 20 (all) when compared with clones without mutations introduced to the FOXA motif (control, as listed in Figure S13). WT represents the observed allelic imbalance in ELOVL2 expression in normal HepG2 cells (n=3).

Figure S18. The effect of AS-gRNA targeting rs953413 coupled with dCas9-effector on ELOVL2 expression. Related to Figure 6. The expression of ELOVL2 was normalized to the expression of ACTB (A) and GAPDH (B), respectively. HepG2 cells transduced with only dCas9-effector virus was employed as the control. **P < 0.01 and ns, not significant calculated by two-tailed Student’s t tests. Error bars, s.d. n = 4 technical replicates.

Figure S19. The expression of ELOVL2 was significantly decreased by mutations introduced to rs953413 by AS-gRNA targeting rs953413 coupled with wide type Cas9. Related to Figure 6. The
expression of ELOVL2 was normalized to the expression of ACTB (A) and GAPDH (B). The expression of ELOVL2 in HepG2 cells transduced with lentiCRISP v2 virus was used as the control. \( n = 12 \) technical replicates. The \( P \) values were calculated by two-tailed Student's \( t \) tests.

Supplemental Tables

Table S3. AS-SNPs in the ELOVL2 locus. Related to Figure 1.

| SNP       | allele       | AF (EUR) | FOXA1 (sc101058) | FOXA1 (sc6553) | FOXA2 (sc6554) |
|-----------|--------------|----------|------------------|----------------|----------------|
|           |              |          | Rep 1 | Rep 2 | Rep1 | Rep2 | Rep1 | Rep2 |
| rs953413  | rs953413[G]  | 0.56     | 22    | 12    | 15   | 15   | 4    | 28   |
|           | rs953413[A]  | 0.44     | 5     | 4     | 14   | 4    | 1    | 9    |
| rs3798713 | rs3798713[G] | 0.58     | 10    | 11    | 17   | 4    | NA   | 16   |
|           | rs3798713[C] | 0.42     | 2     | 1     | 5    | 3    | NA   | 2    |
| rs17675073| rs17675073[G]| 0.86     | 0     | 2     | 3    | 1    | NA   | 2    |
|           | rs17675073[A]| 0.14     | 9     | 9     | 13   | 2    | NA   | 4    |

Note: The number of uniquely mapped reads covering each allele in ChIP-seq was reported for each AS-SNP. NA, no reads covered. AF, allele frequency in European population.

Transparent Methods

Allelic imbalance in chromatin binding of the rs953413 and rs3798713 region in ChIP-seq experiments

The bam files for each TF mapped to regions where AS-SNPs are located were acquired from the ENCODE project (The Encode Project Consortium, 2012). Duplicate reads were removed with picard MarkDuplicates (http://broadinstitute.github.io/picard). Aligned reads with mismatch other than the SNP were discarded with bamtools (Barnett et al., 2011). The reads mapped to each allele were separated with splitSNP (https://github.com/astatham/splitSNP) and counted.

Expression plasmids construction

The human FOXA1 gene was amplified from HepG2 cDNA and cloned into pCDNA3.1 through SLiCE cloning method (Zhang et al., 2012). The ORF clone of FOXA2 was purchased from Sino Biological (HG14174-G). Plasmid pCDNA3.1-FOXA2 was constructed by inserting the coding region of FOXA2 into pCDNA3.1 digested with HindIII and EcoRV through SLiCE cloning method (Zhang et al., 2012). FR_HNF4A2 was a gift from Gerhart Ryffel (Addgene plasmid # 31100) (Thomas et al., 2004). Plasmid
pCDNA3.1-HNF4α was constructed by inserting the coding sequence of HNF4α isoform 2 digested with KpnI and NotI from FR_HNF4A2 into pCDNA3.1 digested with the same enzymes. All the plasmids are validated by Sanger sequencing. The detailed primer sequences for each expression construct is listed in Table S5.

Luciferase constructs and reporter assay

Plasmid pSV40 was constructed by transfer the SV40 promoter sequence from pGL3-promoter vector into pGL4.10 (Promega) through BglII and HindIII double digestion. The 287-bp (chr6: 11,008,440-11,008,726; hg19) fragment containing both rs3798713 and rs17675073 was used for enhancer validation. The 529-bp (chr6: 11,012,583-11,013,111; hg19) rs953413-centered region was also selected for enhancer validation based on the ChIP-seq signals for different TFs from the ENCODE project (Figure S4). The regions were amplified from HepG2 genomic DNA and inserted upstream of either the minimal promoter (MP) sequence of pGL4.23 (Promega) or the SV40 promoter sequence of pSV40 to test their enhancer activities. To identify the ME region encompassing rs953413, a series of truncation and deletion luciferase constructs were generated by SLiCE cloning method (Zhang et al., 2012). A three fragments In-fusion cloning system (Takara) with mutations introduced through primer sequences was employed to introduce desired mutations into luciferase constructs. The detailed primer information and cloning method for each construct is listed in Table S4. All the resulting plasmids were verified by Sanger sequencing.

HepG2 cells were plated one day before transfection in 96-well plates. The confluency was 50-70% on transfection. For normal luciferase assay, each well was transfected with 0.3 µl X-tremeGENE HP DNA transfection reagent (Roche) and 100 ng of experimental firefly luciferase reporter plasmid, and 1 ng of pGL4.74 renilla luciferase reporter vector as internal control for monitoring transfection and lysis efficiency. For luciferase assay overexpressing HNF4α in Figure 4A and 4B, each well was transfected with 0.3 µl X-tremeGENE HP DNA transfection reagent (Roche), 40 ng of firefly luciferase reporter plasmid, 50 ng pCDNA3.1-HNF4α and 10 ng of pRL-MP described earlier (Pan et al., 2017). For other cotransfection experiments, each well was transfected with 0.3 µl X-tremeGENE HP DNA transfection reagent (Roche), 50 ng of firefly luciferase reporter plasmid, 50 ng of expression plasmid and 1 ng of pGL4.74. For experiments cotransfecting HNF4α and FOXA1 in Figure 5A and cotransfecting HNF4α and FOXA2 in Figure S10, 25 ng of each expression plasmid was used with plasmid pCDNA3.1 used as control.

Cells were harvested 24 h after transfection and assayed with the Dual-Luciferase Reporter Assay System (Promega) on an Infinite M200 pro reader (Tecan). All the results are expressed directly as the ratio of firefly luciferase activity from experiment plasmids to renilla luciferase activity from control plasmids. All the luciferase experiments came from two independent transfections i.e., independent plasmid preparations and transfections each with three technical replicates. Luciferase values are expressed as
averages with error bars representing standard deviations (s.d) from all technical replicates and statistical analyses were performed by two-tailed Student’s t tests.

Cell culture

HepG2 cells were originally purchased from the American Type Culture Collection (ATCC) and maintained in RPMI1640 basal medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Human PLC/PRF/5 cells were purchased from European Collection of Authenticated Cell Cultures (ECACC 85061113) and maintained in high glucose DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate. 293T cells were grown in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 500 µg/ml Geneticin. All the cells were also supplemented with 100 units of penicillin and 100 µg of streptomycin per 1 ml of culture medium.

Lentiviral-mediated gene knockdown

Both shRNA and artificial miRNA expression cassettes were utilized to knockdown target genes. The shRNA sequence was directly synthesized and inserted into pGreenPuro shRNA expression lentivector (Si505A-1, System Biosciences) digested with BamHI and EcoRI. The artificial miRNA precursor was constructed from the natural miR-124-1 precursor sequence introducing two suitable restriction sites recognized by EcoRV and EcoRI, respectively (Liang et al., 2012). The constructed artificial miRNA precursor was inserted downstream of the puromycin resistance gene through SLiCE cloning method. The target sequences for FOXA1 knockdown are GATGTGTAGACATCCTCCGTATATT and GGCGTACTACCAAGGTGTGTA. For HNF4α knockdown, the target sequences are GAACCACATGTACTCCTGCAGATT and TCAGCACTCGAAGGTCAAGCTAT. The detailed primer information for each lentiviral construct is listed in Table S5.

Lentivirus was produced in 293T cells by transfecting the lentiviral plasmid together with packaging plasmids pLP1, pLP2 and envelope plasmid pLP/VSVG (Life Technologies) using polyethylenimine (Polysciences) following the manufacturer’s instructions. Cells were plated in 24-well plates and transduced with virus supernatant together with sequabrene (Sigma) at final concentration of 8 µg/ml. The cell lines with double knockdown of FOXA1 and HNF4α were achieved by sequential or simultaneous transduction of virus knocking down FOXA1 and HNF4α. The cell lines with target gene knockdown were selected by puromycin (Life Technologies) at concentration of 1 µg/ml for HepG2 cells and 4 µg/ml for PLC/PRF/5 cells. The selected cells were maintained with 0.5 µg/ml of puromycin and regularly passaged for further analysis.

Quantitative real time PCR
At least three replicates were studied by quantitative real time PCR (qPCR) on cDNA samples and on genomic DNA samples from ChIP experiments. List of primers used in this study is shown in Table S6. The qPCR reactions were performed with JumpStart Taq ReadyMix (Sigma) coupled with EvaGreen dye (Biotium). For gene expression analysis, equal number of cells (8 × 10^5 for HepG2 cells and 2.5 × 10^5 for PLCR/PRF/5 cells) were plated in 12-well plates 24 h before harvesting. Total RNA was extracted from cells with TRIzol (Life Technologies) according to the manufacturer’s instructions. A total of 1 µg total RNA was reverse transcribed into cDNA with Maxima First Strand cDNA synthesis kit (Life Technologies). The expression was normalized to three control transcripts including RSP18, ACTB and GAPDH. Statistical analyses were carried out by two-tailed Student’s t tests.

For ChIP experiments, a standard curve was generated for each target with serial-diluted input DNA as templates. The amounts of DNA pulled down for each target was first normalized to the input based on the standard curve. Then the relative enrichment of target DNA was displayed as fold changes over the negative control region GDCHR12, located in one gene desert region on chromosome 12. The promoter region of HNF1α (HNF1α-pro) was used as the positive control. All the primer sequences used for qPCR analysis are listed in Table S6.

Allele-specific quantitative PCR

Primers for AS-qPCR were designed based on mismatch amplification mutation assay (MAMA) to accurately quantify single nucleotide mutations (Cha et al., 1992; Li et al., 2004). Allele-specific amplification of the rs953413 region was employed to validate the allelic imbalance in chromatin binding of both TFs and H3K27ac in ChIP experiments. The exonic rs2295601 AS-qPCR was used to evaluate the allelic imbalance in ELOVL2 expression. The PCR reactions were carried out with JumpStart Taq ReadyMix (Sigma) coupled with EvaGreen dye (Biotium). The specificity of the AS-qPCR primers was validated with genomic DNA from different samples as templates from the Excellence of Diabetes Research in Sweden (EXODIAB) biobank and shown in Figure S7. The detailed primer sequences used in AS-qPCR are listed in Table S6.

Chromatin immunoprecipitation

ChIP experiments were carried out as previously described with modifications (Blecher-Gonen et al., 2013; Pan et al., 2017). Cells were cultured in T175 flask until reaching confluency of >80% and crosslinked with 1% formaldehyde on a shaking platform for 10 min at room temperature and quenched with 125 mM glycine for 5 min. Cells were collected, washed twice with ice-cold PBS and resuspended in cell lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl and 0.2% NP-40) supplemented with protease inhibitor (Roche) to isolate nuclei. RIPA buffer (1× PBS, 1%NP-40, 0.1% SDS, 0.5% sodium deoxycholate and 0.004% sodium azide) supplemented with protease inhibitor was used to isolate the cross-linked
chromatin from nuclei. The isolated chromatin was sonicated to an average size of 250 bp using the Bioruptor Pico sonication device (Diagenode). An aliquot was saved each time to be subjected to DNA extraction and used as input. The leftover was used for ChIP assay.

For ChIP experiments with antibody against histone modification H3K27ac, 4 µg of antibody from Abcam (ab4729) together with 40 µl of Dynabeads protein G (Life Technologies) was added simultaneously into sonicated chromatin from 1 × 10^6 cells and incubated on a rotating platform overnight at 4°C. For ChIP experiments against TFs, chromatin from 3 × 10^6 cells was first precleared by incubating with 30 µl Dynabeads for 2 h at 4°C. The supernatant was separated and incubated overnight at 4°C with 8 µg of antibody against FOXA1 (ab5089, Abcam), FOXA2 (SC-6554 for HepG2 and SC-374376 for PLC/PRF/5 due to discontinuation of antibody used in HepG2 cells, Santa Cruz Biotechnology) or HNF4α (SC-6564X for HepG2 cells and SC-374229 for PLC/PRF/5 due to discontinuation of antibody used in HepG2 cells, Santa Cruz Biotechnology). Normal rabbit IgG (12-370, Millipore) was also included to check the background of antibody nonspecific binding in each batch of experiments. The immune complexes were captured by incubation with 75 µl of Dynabeads protein G at room temperature for 1 h. For all ChIP assays, a series of washing steps was applied to Dynabeads protein G after immunoprecipitation including four times of washing with RIPA buffer, two times of washing with ChIP wash buffer (10 mM Tris–HCl, pH 8.0, 0.25 M LiCl, 10 mM EDTA, 1% NP-40 and 1% sodium deoxycholate) and once with TE buffer. The DNA–protein complexes were eluted in direct elution buffer (10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.3 M NaCl and 0.5% SDS) and together with the previous saved sonicated lysates to be used as input were treated with RNase A and proteinase K respectively and incubated at 65°C overnight to reverse crosslink. DNA was purified with Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer’s instructions, eluted in 10 mM Tris–HCl (pH 8.0) and ready to be analyzed by qPCR. Input DNA was quantified by Nanodrop 2000 (Life Technologies) and serial diluted to be used as templates for setting up stand curves of different primers targeting candidate regions.

**CRISPR/Cas9-mediated genome editing**

LentiCRISPR v2 (Addgene plasmid # 52961) was a gift from Feng Zhang (Sanjana et al., 2014). In order to knock out the ME element, two gRNAs adjacent to the ME element were designed. The expression of the two gRNAs are driven by human and mouse U6 promoter, respectively. The expression cassette is inserted in lentiCRISPR v2 digested with BsmBI by In-Fusion cloning system (Takara). The AS-gRNA targeting each allele of rs953413 was directly synthesized and inserted into lentiCRISPR v2 digested with BsmBI through T4 ligation. The identity of all the constructs were validated by Sanger sequencing. The detailed primer information is listed in Table S5. The lentivirus was produced in 293T cells and transduced into HepG2 cells coupled with sequabrene (Sigma) at final concentration of 8 µg/ml in 12-well plate. The positive cells were selected by puromycin (Life Technologies) at concentration of 1 µg/ml. Individual colonies were generated by splitting the positive cells to 0.5~3 cells per well in 96-well
plates and culturing in normal medium until single colony is formed. The genomic DNA for each single colony in 96-well plate is extracted by QuickExtract DNA Extraction Solution (QE09050, Lucigen). The introduced mutations for each colony are verified by both direct PCR sequencing and TA cloning followed by Sanger sequencing with the primers listed in Table S6. The selected colonies are passed regularly until reaching T75 flask and ready for expression study.

**Transactivation and transrepression of the rs953413 locus through dCas9-effector coupled with rs953413 AS-gRNA**

Lentiviral plasmid lenti-EF1a-dCas9-KRAB-Puro (Addgene plasmid # 99372), lenti-EF1a-dCas9-VP64-Puro (Addgene plasmid # 99371) and lentiGuide-Hygro-eGFP (Addgene plasmid # 99375) were gifts from Kristen Brennand (Ho et al., 2017). The AS-gRNA targeting each allele of rs953413 was directly synthesized and inserted into lentiGuide-Hygro-eGFP digested with BsmBI through T4 ligation. The HepG2 cells stably expressing dCas9-KRAB and dCas9-VP64 were acquired by lentiviral transduction followed by puromycin selection at 1 µg/ml. The cells expressing dCas9-effector were further transduced with lentivirus expressing AS-gRNA targeting either the A allele or the G allele of rs953413 and selected with medium containing 1 µg/ml puromycin and 0.2 mg/ml of hygromycin B (Life Technologies) until ready for expression study.

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