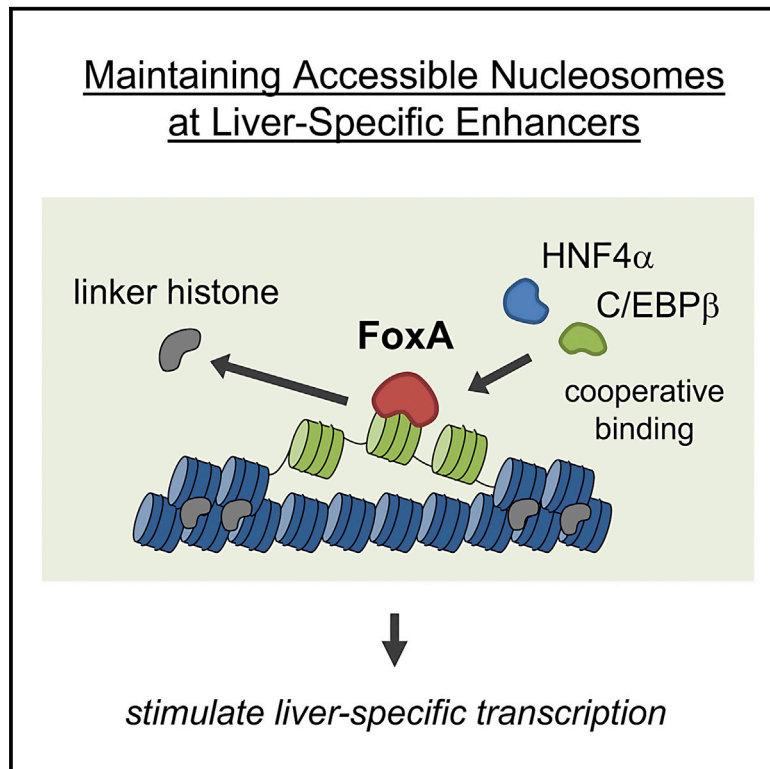


The Pioneer Transcription Factor FoxA Maintains an Accessible Nucleosome Configuration at Enhancers for Tissue-Specific Gene Activation

Graphical Abstract



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In Brief

Using low- and high-MNase sequencing with core histone mapping, Iwafuchi-Doi et al. reveal that tissue-specific enhancers retain accessible nucleosomes more than promoters and ubiquitous enhancers in mammalian chromatin. The pioneer factor FoxA displaces linker histone, thereby keeping nucleosomes accessible and allowing other liver-specific transcription factors to bind and stimulate gene activation.

Highlights

- Liver-specific enhancers retain accessible nucleosomes more than ubiquitous enhancers
- FoxA binding displaces linker histone and keeps nucleosomes accessible
- FoxA2 is enriched near the dyad axis of accessible nucleosomes with other liver TFs
- FoxA-bound nucleosomes at enhancers stimulate liver gene activation

Accession Numbers

GSE57559



The Pioneer Transcription Factor FoxA Maintains an Accessible Nucleosome Configuration at Enhancers for Tissue-Specific Gene Activation

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<http://dx.doi.org/10.1016/j.molcel.2016.03.001>

SUMMARY

Nuclear DNA wraps around core histones to form nucleosomes, which restricts the binding of transcription factors to gene regulatory sequences. Pioneer transcription factors can bind DNA sites on nucleosomes and initiate gene regulatory events, often leading to the local opening of chromatin. However, the nucleosomal configuration of open chromatin and the basis for its regulation is unclear. We combined low and high levels of micrococcal nuclease (MNase) digestion along with core histone mapping to assess the nucleosomal configuration at enhancers and promoters in mouse liver. We find that MNase-accessible nucleosomes, bound by transcription factors, are retained more at liver-specific enhancers than at promoters and ubiquitous enhancers. The pioneer factor FoxA displaces linker histone H1, thereby keeping enhancer nucleosomes accessible in chromatin and allowing other liver-specific transcription factors to bind and stimulate transcription. Thus, nucleosomes are not exclusively repressive to gene regulation when they are retained with, and exposed by, pioneer factors.

INTRODUCTION

Chromatin consists of a fundamental repeating unit, the nucleosome, which contains genomic DNA wrapped around an octamer of the core histones H2A, H2B, H3, and H4. Nucleosome organization provides steric constraints on how transcription factors (TFs) can bind gene regulatory sequences and thereby impacts diverse processes, from cell differentiation to disease progression (Jiang and Pugh, 2009; Voss and Hager, 2014). Although most TFs cannot access nucleosomal DNA on their own, a subset of TFs possess a special ability to engage their target sites on nucleosomal DNA and are thus referred to as

“pioneer” factors (Iwafuchi-Doi and Zaret, 2014; Soufi et al., 2015). In higher eukaryotes, gene expression is regulated by the coordinated action of enhancers and promoters (Levine et al., 2014). Active enhancers and promoters share diverse features, including TF occupancy, open chromatin, as seen by DNase I hypersensitivity (Thurman et al., 2012), and active histone modifications (Creighton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Shen et al., 2012). In mammalian genomes, enhancers tend to be active in a tissue-specific manner, while promoters are more likely to be used in a ubiquitous fashion (Heintzman et al., 2009; Thurman et al., 2012). Diverse lines of evidence indicate that the open chromatin structure at promoters primarily consists of a nucleosome- and histone-free region established by DNA sequence, general TFs, chromatin remodelers, and the basal transcription machinery (Floer et al., 2010; Hughes et al., 2012; Jiang and Pugh, 2009; Rhee et al., 2014; Struhl and Segal, 2013).

In contrast, there are conflicting reports on the presence of nucleosomes at active enhancers. Single-locus studies with titrations of enzyme probes, sequential chromatin immunoprecipitation (ChIP), and in vivo footprinting indicate an open chromatin structure at a liver-specific enhancer that retains phased nucleosomes, bound by the pioneer factor FoxA and other TFs (Chaya et al., 2001; McPherson et al., 1993). A genome-wide micrococcal nuclease (MNase) mapping study showed that at least half of FoxA2 binding sites were nucleosome-free, while the remainder of the sites were with MNase-resistant fragments (Li et al., 2011). Yet, the prevailing genome-wide view, based on MNase-sequencing (MNase-seq) mapping studies, is that DNase-hypersensitive, open chromatin structures at enhancers are essentially nucleosome-free (Chai et al., 2013; Gaffney et al., 2012; Kim and Shiekhhattar, 2015). Pioneer factors are candidates to modulate nucleosome configuration, since in vitro studies showed that FoxA can open a local domain of chromatin without the help of an ATP-dependent chromatin remodeler (Cirillo et al., 2002; McPherson et al., 1993; Shim et al., 1998). Thus, the typical nucleosome configuration at enhancers, how it is regulated in vivo, and how it associates with tissue-specific or ubiquitous gene regulation remain unclear.

Nucleosome positions have been mapped by digesting chromatin with MNase. MNase initially cuts chromatin between nucleosomes to generate 180- to 200-bp fragments from mammalian chromatin; further digestion trims the DNA to 147 bp and then degrades the nucleosome core particle itself (Axel, 1975). Thus, in a high-level digestion reaction, nucleosomes in open chromatin are destroyed, whereas nucleosomes in closed chromatin are not. Most genome-wide nucleosome mapping studies apply high-level MNase digestion to maximize mononucleosome yield in bulk chromatin (e.g., Chai et al., 2013; Gaffney et al., 2012) and thus could destroy nucleosomes in open chromatin. A prior nucleosome mapping study, using relatively high levels of MNase digestion, reported no differences in nucleosome occupancy at selected sites in the presence and absence of FoxA1 and FoxA2, possibly because nucleosomes at open chromatin regions had been missed (Li et al., 2011). A recent study, using substantially lower levels of chromatin digestion with methidium-propyl-EDTA coupled with core histone ChIP, revealed more apparent nucleosome retention than had been revealed by prior high-level MNase studies (Ishii et al., 2015). In yeast, low levels of MNase digestion recovered DNA fragments within previously determined “nucleosome-free” regions (200 bp upstream of the transcription start site [TSS]) in a subset of promoters, in addition to within the -1 nucleosome position (upstream of the nucleosome-free region) were named “fragile nucleosomes” (Rhee et al., 2014; Weiner et al., 2010; Xi et al., 2011). However, a recent high-resolution core histone mapping study indicates nucleosome-free regions at promoters are indeed histone-free (Rhee et al., 2014).

Here, we sought to assess the occupancy and accessibility of nucleosomes at tissue-specific and ubiquitous regulatory sequences in a native animal tissue and how nucleosome accessibility and its consequences are controlled. We mapped nucleosomes in adult mouse liver by low-level and high-level MNase digestion in conjunction with mapping core histones. We found that, contrary to the predominant assumption in the field, active, tissue-specific enhancers retain MNase-accessible nucleosomes significantly more than promoters and ubiquitous enhancers. Furthermore, FoxA2 is enriched near the dyad axis of accessible nucleosomes and displaces linker histones, thereby keeping nucleosomes accessible at liver-specific enhancers and helping other TFs to bind. We suggest that nucleosomes are not exclusively repressive to gene regulation when they are retained with, and exposed by, pioneer factors to stimulate tissue-specific gene activation.

RESULTS

Low-MNase Digestion Preferentially Recovers Nucleosome-Size Fragments in Open Chromatin Regions

We first tested how the level of MNase digestion affects the recovery of nucleosomal-size DNA fragments at the *Alb1* enhancer, which is DNase hypersensitive in liver, but not hypersensitive in spleen (Chaya et al., 2001; McPherson et al., 1993). We prepared native nuclei from adult mouse liver and spleen in a buffer containing 10 mM NaCl to prevent salt-dependent dissociation of nucleosome cores (Jin and Felsenfeld, 2007), and we digested

the nuclei with a low concentration of MNase (0.5–1 U/ml) as well as a high concentration (20 U/ml). The low-MNase digestion generated large polynucleosomal fragments with a small fraction of mononucleosomal-size DNA ($\sim 1.5\%$ – 2.5% of input chromatin; average size, 175 bp), while the high-MNase digestion generated predominantly mononucleosomal-size DNA ($\sim 25\%$ – 30% of input chromatin; average size, 160 bp) (Figure 1A). The latter is similar to that seen in most conventional MNase digestion for nucleosome mapping. We isolated four sizes of DNA fragments from both digests: sub-nucleosomal (25–140 bp) DNA, mononucleosomal (140–200 bp) DNA, 1.5 nucleosome (nuc) DNA (200–260 bp), and dinucleosomal (260–400 bp) DNA (Figure S1A). We then assessed the recovery of the DNase-hypersensitive N1 nucleosome, compared to the adjacent non-hypersensitive N2 nucleosome, at the liver-specific *Alb1* enhancer (Chaya et al., 2001; McPherson et al., 1993) by qPCR (Figure S1B). The low-MNase digestion enriched for the hypersensitive N1 nucleosome in the mononucleosomal-size fraction from liver, but not from spleen, and modestly enriched for the N2 nucleosome (Figure S1C). In contrast, the conventional high-MNase digestion markedly degraded the hypersensitive N1 nucleosome (Figure S1C). Thus, the low-MNase digestion preferentially recovers MNase-accessible and DNase-hypersensitive nucleosomal-size DNA, whereas conventional, high-MNase digestion degrades it.

Liver-Specific Enhancers Retain MNase-Accessible Nucleosomes More Than Promoters and Ubiquitous Enhancers

We mapped the genome-wide distribution of MNase-resistant fragments (140–200 bp) from two biological replicates of the low-MNase digestion (total >195 million low-MNase-seq paired-end reads) and the high-MNase digestion (total >270 million high-MNase-seq paired-end reads) in mouse liver (Table S1). Shen and colleagues predicted tissue-specific and ubiquitous (active in many cell types) enhancers based on p300 binding distal to TSSs, along with H3K4me1, but not H3K4me3, in the mouse genome (Shen et al., 2012). We further filtered these enhancers with H3K27ac peaks (ENCODE: GSM1000140), because H3K27ac predominantly marks active regulatory sequences, while H3K4me1 marks both poised and active enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011), and we centered the enhancers by DNase-hypersensitive sites (ENCODE: GSM1014195), where TFs are bound.

Two examples of active promoters and enhancers, respectively (red blocks), showed greater low-MNase-seq signals (green tracks) than high-MNase-seq signals (purple tracks) (Figures 1B and S1D), indicating the presence of MNase-accessible chromatin. To test whether the low-MNase-seq signals represent nucleosomes, we performed ChIP-qPCR for core histones with crosslinked and highly sonicated chromatin in order to avoid an MNase digestion bias. Notably, the two examples of liver-specific enhancers were markedly less depleted for core histones H2B and H3 than the active promoters (Figures 1C and S1E), suggesting that low-MNase signals at liver-specific enhancers represent nucleosomes, but those at promoters do not. Genome-wide analysis also confirmed greater low-MNase enrichment than high-MNase-seq signals over active regulatory

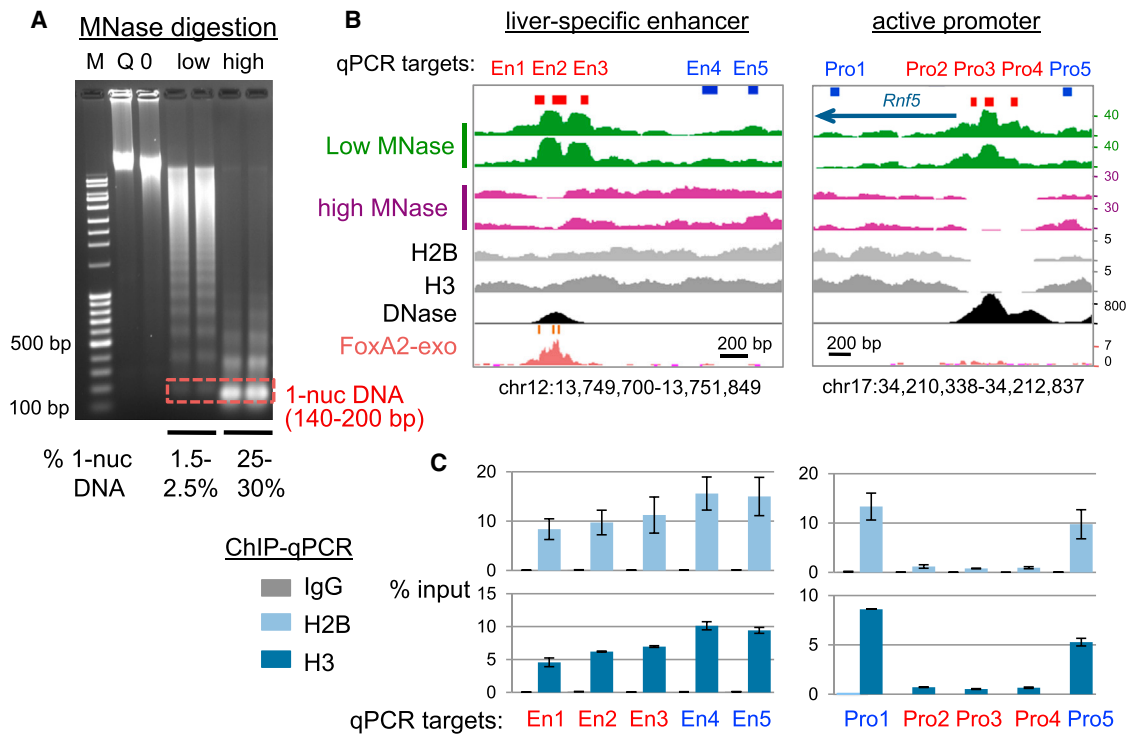


Figure 1. Liver-Specific Enhancers Retain MNase-Accessible Nucleosomes

(A) Marker (M), MNase-untreated controls, Q (not warmed) and 0 (warmed), and low- or high-MNase digested DNAs on an agarose gel.

(B) Signal tracks of MNase/ChIP/DNase-seq reads; two biological replicates for MNase-seq. Red boxes, sites of low MNase tag enrichment tested by qPCR in (C); blue boxes, control sites.

(C) Core histone ChIP-qPCR enrichment (means \pm SD of two biological replicates).

See also Figure S1.

sequences (Figure 2A for averaged signal; p value $< 2.2e-16$; and Figure 2B for individual signals).

Furthermore, we found that low-MNase-seq signals were most enriched at liver-specific enhancers (central 200 bp), compared to ubiquitous enhancers (central 200 bp) and active promoters (upstream 200 bp of TSS) (Figure 2A; p value $< 2.2e-16$). Since the number of ubiquitous enhancers is much smaller ($n = 383$) than the other groups, we randomly sampled 383 liver-specific enhancers in order to compensate for the difference. We confirmed that the sampled enhancers have similar MNase profiles as all liver-specific enhancers, as well as a higher enrichment of the low-MNase-seq signals than at ubiquitous enhancers (p value $< 2.2e-16$) (data not shown; see Supplemental Experimental Procedures, Meta-locus plots), suggesting that our findings are not due to the difference of the number of sites tested. Notably, genome-wide ChIP-seq showed that the liver-specific enhancers (central 200 bp) were slightly depleted for core histones H2B and H3, but significantly less so than the ubiquitous enhancers (central 200 bp; p value 0.005 for H3 and $5.3e-12$ for H2B) and markedly less depleted for the histones than were the active promoters (upstream 200 bp of TSS; p value $< 2.2e-16$ for H2B and H3) (Figures 2C and S2C).

We also curated active promoters into liver-specific and ubiquitous promoters based on RNA polymerase II binding patterns (Shen et al., 2012), and the majority of promoters appeared to

be active in other tissues, designated as ubiquitous ($n = 5,010$), rather than liver specific ($n = 169$). Although the liver-specific promoters had more low-MNase signal and less histone depletion, compared to ubiquitous promoters, they were significantly more depleted of core histones than liver-specific enhancers (p value $< 2.2e-16$ for H3 and H2B) (Figures S2A and S2B). Since the liver-specific enhancers are more stably or frequently occupied by nucleosomes in accessible chromatin, in contrast to ubiquitous enhancers and active promoters, we refer to the former nucleosomes as “exposed” or “accessible.” Previous studies had reported low-MNase signal enrichment at subset of active promoters (upstream 200 bp of TSS) in yeast, which they designated as “fragile nucleosomes” (Weiner et al., 2010; Xi et al., 2011). However, we found that such low-MNase enrichment at active promoters in mammalian tissue can represent primarily a histone depleted state, likely by the stochastic production of mono-nucleosomal size fragments even on nucleosome-free DNA. Regardless, there is a marked difference in histone retention between active liver-specific enhancers and ubiquitous enhancers and promoters.

Histone Modifications and Variants that Do Not Correlate with Accessible Enhancer Nucleosomes

To address which factors are associated with accessible nucleosomes in native liver, we compared several active chromatin

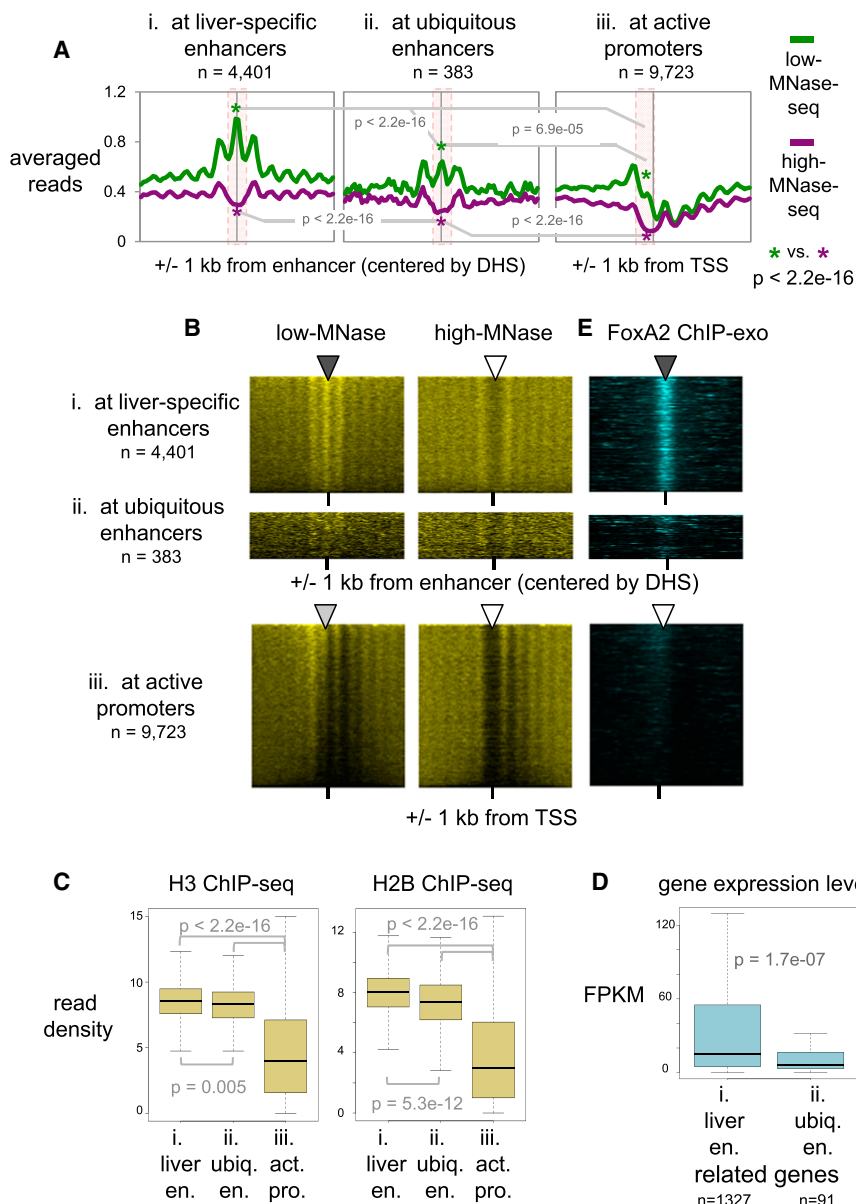


Figure 2. Liver-Specific Enhancers Retain MNase-Accessible Nucleosomes Genome-wide

(A) Averaged profiles of low- and high-MNase-seq signal enrichments at liver-specific enhancers, ubiquitous enhancers, and active promoters. p values by Wilcoxon rank-sum test at central 200 bp of enhancers and 200 bp upstream of the active TSS (pink box area).

(B and E) Heatmaps of MNase-seq and FoxA2 ChIP-exo at liver-specific enhancers, ubiquitous enhancers, and active promoters, rank ordered by low-MNase-seq read density.

(C) Box and whisker plots show H3 and H2B ChIP-seq signal enrichment at the central 200 bp of liver-specific and ubiquitous enhancers and 200 bp upstream of the active TSS. p value by Wilcoxon rank-sum test.

(D) Gene expression levels of target genes of liver-specific and ubiquitous enhancers, based on predicted enhancer-promoter pairs (Shen et al., 2012).

See also Figure S2.

(Shen et al., 2012). Based on this prediction and liver RNA-sequencing data (ENCODE: ENCSR000AJU), we assessed the gene expression levels of target genes of liver-specific and ubiquitous enhancers. Remarkably, the liver-specific enhancers, enriched for accessible nucleosomes, drove higher gene expression levels than the ubiquitous enhancers (Figure 2D), even though the former exhibited lower “active” modification states. Furthermore, when we compared the same number of enhancers from the liver-specific and ubiquitous group exhibiting similar ranges of active H3K27ac enrichment, we still observed higher enrichment of low-MNase and core histone signals at the liver-specific enhancers than at the ubiquitous enhancers (Figure S2E). These results indicate that active histone modifications

features. Surprisingly, the active histone modification H3K27ac (ENCODE: GSM1000140) and the enhancer histone modification H3K4me1 (ENCODE: GSM769015) were not markedly enriched with accessible nucleosomes at liver specific enhancers (compare Figures 2A and S2D). Similarly, histone variants H2A.Z (Menet et al., 2014) (GSE47143) and H3.3 (Bargaje et al., 2012; ERR234277) did not show proportional enrichment directly over accessible nucleosomes at liver-specific enhancers, though there was modest enrichment nearby (compare Figures 2A and S2D).

Since the accessible nucleosomes in liver-specific enhancers were in a low-modification state, we tested whether the expression levels of the corresponding target genes was also low. Shen and colleagues developed an algorithm to detect co-regulated promoters–enhancer clusters and effectively predicted enhancer-promoter pairs in various tissues, including adult liver

known to be enriched at enhancers (largely from cell line studies) do not drive nucleosome accessibility or target gene expression at active enhancers in adult liver tissue. Our results are consistent with recent findings that developmentally regulated genes in embryos and tissue-specific genes show much lower enrichment for active histone modifications, compared to ubiquitously expressed genes, even though the former genes are expressed at comparable or higher levels than ubiquitous genes (Pérez-Lluch et al., 2015).

The Pioneer Factor FoxA2 Is Enriched at the Dyad Axis of Accessible Nucleosomes, Along with Other Liver-Enriched TFs

We next assessed the relationship between binding of the pioneer factor FoxA, which regulates many liver-specific genes

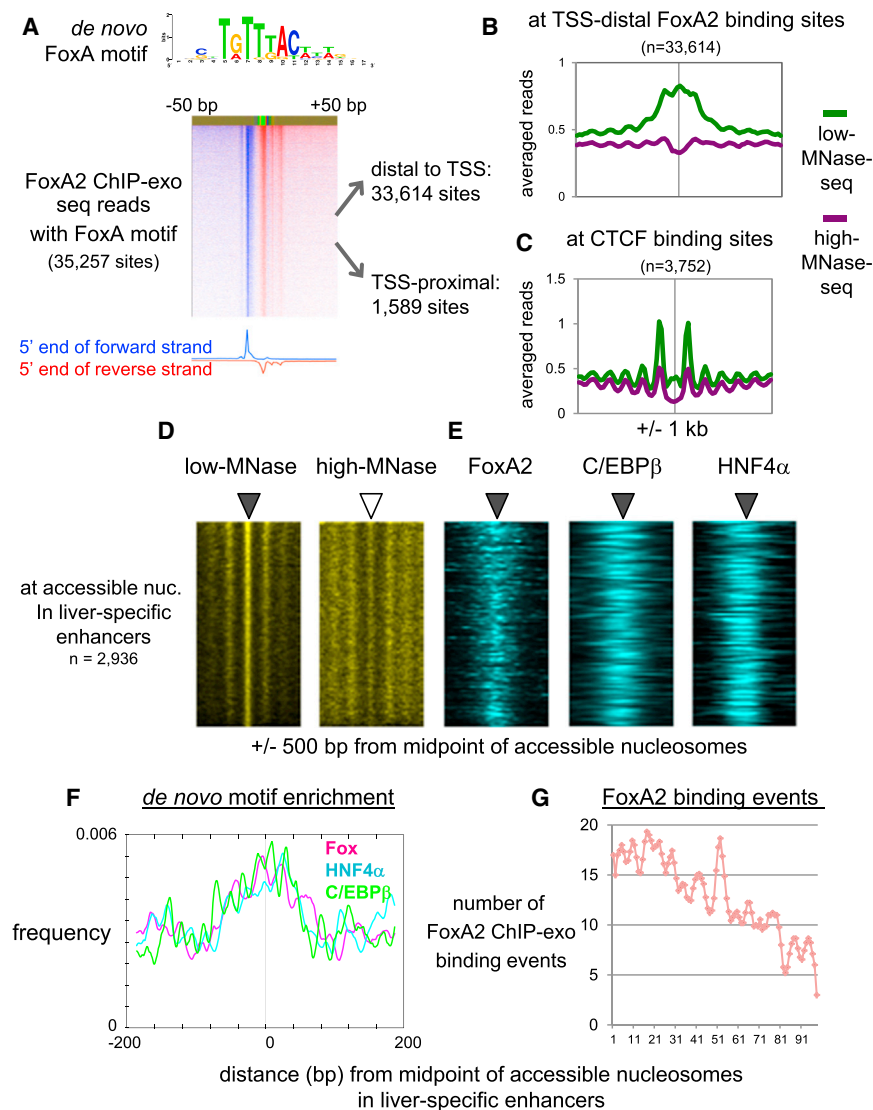


Figure 3. Pioneer Factor FoxA2 and Liver TFs Are Co-localized at Accessible Nucleosomes in Liver-Specific Enhancers

(A) FoxA2 ChIP-exo tag 5' end distribution centered by *de novo* FoxA motif midpoint and sorted by FoxA2 occupancy level. (B and C) Averaged profiles of low- and high-MNase-seq at TSS-distal FoxA2 binding sites (B) and at CTCF binding sites (C) overlapping with H3K27ac peaks. (D and E) Heatmaps of MNase-seq and FoxA2 ChIP-exo, C/EBP β , and HNF4 α ChIP-seq at MNase-accessible nucleosomes in liver-specific enhancers, rank ordered by low-MNase-seq read density. (F) Profiles of *de novo* motifs enrichment relative to midpoint of accessible nucleosome in enhancers. (G) Histogram of FoxA2 ChIP-exo binding locations relative to midpoint of accessible nucleosomes. The midpoint approximating the position of the respective nucleosome dyad axis. See also Figure S3.

enhancers, but not at ubiquitous enhancers and active promoters (Figure 2E). We compared our MNase and core histone enrichments at the FoxA2 binding sites with such enrichments at CTCF binding sites (ENCODE: GSM918715), which are known to be nucleosome-free and flanked by well-positioned nucleosomes (Carone et al., 2014), in active chromatin (as assessed by association with H3K27ac peaks). We found that both low-MNase and core histone signals were much more enriched at FoxA2 binding sites compared with CTCF binding sites (Figures 3B, 3C, and S3A; p value < 2.2e-16). This supports our finding that the accessible nucleosome configuration at FoxA2 binding sites represents nucleosomes rather than nucleosome-free DNA, with the latter seen at CTCF sites.

To obtain insight into which factors associate with nucleosomes in an unbiased manner, we conducted *de novo* motif analysis of these regulatory sequences. We found that the ubiquitous enhancers were enriched for the motifs of ubiquitous TFs (Sp1 and Ets factors) and Fox (Figure S3B). The liver-specific enhancers were enriched for the Fox motif, which is very similar to the *de novo* motif from our FoxA2 ChIP-exo analysis, and also enriched for the motifs of other liver TFs (HNF4 α , C/EBP, and Onecut) and certain ubiquitous TF motifs (NFI and STAT) (Figure S3B). The ubiquitous factor Sp1 recruits the SWI/SNF chromatin-remodeling complex to facilitate chromatin remodeling and transcriptional activation (Kadam and Emerson, 2003), consistent with Sp elements occurring at more nucleosome-depleted ubiquitous enhancers (Figure S3B).

Next, we analyzed how the *de novo* motifs were localized relative to the apparent dyad axis of the accessible nucleosomes at

(Li et al., 2012), and nucleosome configuration *in vivo*. Since FoxA1 and FoxA2 exhibit quantitatively similar overlap of binding to many target sites and both factors are, for the most part, functionally redundant in adult liver (Bochkis et al., 2012; Watts et al., 2011), here we focused on FoxA2. We identified 35,257 FoxA2 binding sites harboring the FoxA binding motif in adult mouse liver, at base-pair resolution, by ChIP with lambda exonuclease digestion followed by sequencing (ChIP-exo-seq) (Rhee and Pugh, 2011; Figure 3A). The vast majority of FoxA2 binding sites (95%; 33,614 sites) were located distal to TSSs of RefSeq genes (> \pm 500 bp distal to TSS), and only a minority of FoxA2 binding sites (4.5%; 1,589 sites) were located proximal to TSSs (< \pm 500 bp proximal to TSS; Figure 3A), suggesting that FoxA2 is primarily involved in gene regulation through distal regulatory regions, i.e., enhancers. Both distal and TSS-proximal FoxA2 peaks mostly overlapped with DHS (ENCODE: GSM1014195) (86% and 96%, respectively). We found that binding of FoxA2 was highly enriched for accessible nucleosomes at liver-specific

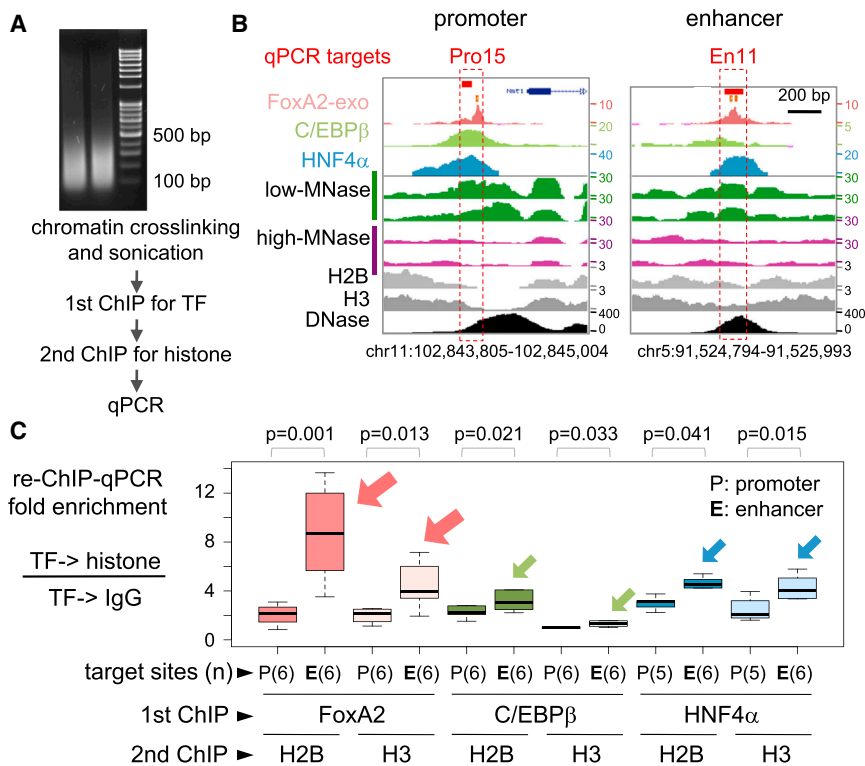


Figure 4. FoxA2 Binds More with Core Histones Than Other Liver TFs at Enhancers

(A) Biological replicates of sonicated liver chromatin on agarose gel.

(B) Examples of re-ChIP-qPCR target sites with MNase/ChIP-seq signals.

(C) Box and whisker plots show re-ChIP-qPCR enrichment of core histone H2B and H3 over immunoglobulin G at the promoter and enhancer target sites. Numbers in parentheses indicate number of sites tested. p value by Wilcoxon rank-sum test.

See also Figure S4.

enhancers. We defined a class of accessible nucleosomes at positions where the 145-bp window counts (centered around MNase-seq fragment midpoints) in the low-MNase-seq data were significantly higher than in high-MNase-seq data (Figure 3D). Ubiquitous enhancers had scattered TF motifs throughout the MNase-accessible sites (Figure S3C). By contrast, the liver-specific enhancers showed FoxA2, C/EBPβ (Jakobsen et al., 2013), and HNF4α (Hoffman et al., 2010) binding events (Figure 3E), and these motifs focused around the nucleosome dyad axis (Figure 3F). Our single-nucleotide-resolution mapping revealed FoxA2 binding events near the nucleosome dyad axis (Figure 3G), as had originally been reported for FoxA at the *Alb1* enhancer by in vivo footprinting studies (McPherson et al., 1993). Altogether, our results suggest that FoxA and other liver-enriched TFs are cooperatively bound to the accessible nucleosomes at liver-specific enhancers.

FoxA2 Binds More with Core Histones Than Other Liver TFs at Enhancers

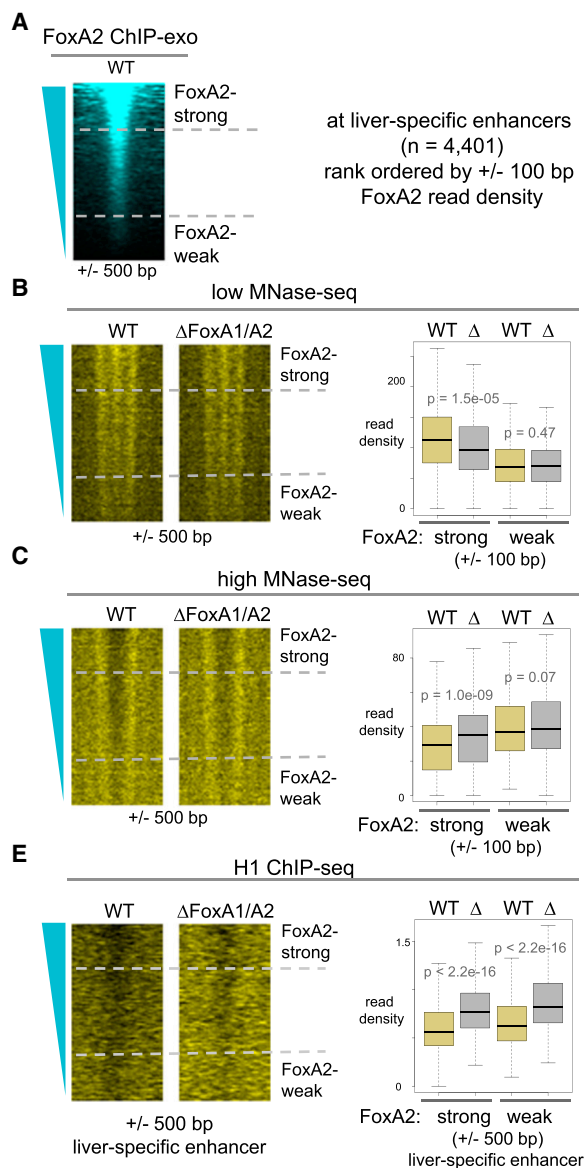
To assess whether FoxA or other liver TFs are bound to accessible nucleosomes in vivo or if there are separate populations of TFs bound to free DNA, we performed sequential ChIP-qPCR (re-ChIP-qPCR) for FoxA2, C/EBPβ, and HNF4α, followed by ChIP for different core histones. To minimize false-positive ChIP signals from large DNA fragments, we fragmented the liver chromatin to about 200 bp by sonication (Figure 4A). We selected qPCR target sites in promoters and enhancers where MNase-accessible regions overlap with TF binding (e.g., Figure 4B). As expected, the first ChIP signals for TFs were enriched at their target sites similarly at promoters and enhancers (Fig-

ure S4). However, the secondary ChIP revealed that FoxA2 was significantly more enriched with core histones at enhancers, compared to promoters (Figure 4C, pink bars; compare P versus E target sites). C/EBPβ and HNF4α were overall less enriched with core histones but still showed a statistically significant enrichment at active enhancers compared to active promoters (Figure 4C, green and blue bars). FoxA2 co-occupancy with core histones was also seen in other studies (Chaya et al., 2001; Li et al., 2011), and here, we show that FoxA2 binds more stably or

FoxA2 Binding Is Required to Keep Nucleosomes Accessible in Chromatin

We addressed whether FoxA can regulate the nucleosome state in vivo using the genome-wide low- and high-MNase method. We deleted *FoxA1* and *FoxA2* in a hepatocyte-specific manner (see Experimental Procedures), purified hepatocytes from the mutant livers, along with livers of wild-type controls, and confirmed depletion of FoxA1 and FoxA2, with residual FoxA3 expression (Figure S5A). Previous MNase-seq data in this study were derived from whole liver, which, considering the polyploidy nature of mature hepatocytes, is 90% comprised of chromatin signals from hepatocytes.

We rank ordered the liver-specific enhancers based on FoxA2 ChIP-exo read density and curated them into the top 25% (FoxA2-strong) and the bottom 25% (FoxA2-weak) FoxA-bound enhancers (Figure 5A). In the wild-type controls, FoxA2-strong enhancers showed a more accessible nucleosome configuration than FoxA2-weak enhancers (Figure 5D, yellow boxes at FoxA2-strong versus FoxA2-weak), which is due to more low-MNase-seq signals (Figure 5B, yellow boxes) and fewer high-MNase-seq signals at FoxA2-strong enhancers (Figure 5C, yellow boxes). In FoxA1/A2 deletion (Δ) mutants, FoxA2-strong enhancers showed a significant reduction of nucleosome accessibility (Figure 5D, left, yellow versus gray boxes), as shown by reduced low-MNase signals (Figure 5B, left, yellow versus gray boxes) and increased high-MNase signals (Figure 5C, left, yellow

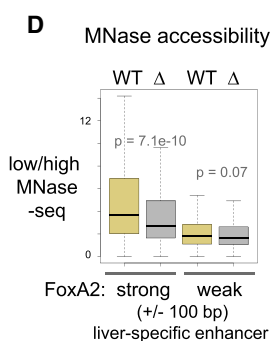


versus gray boxes). However, FoxA2-weak enhancers and silent enhancers in liver (e.g., heart-specific enhancers; Shen et al., 2012), where FoxA did not bind, showed marginal or no effects on nucleosome accessibility in the mutants (Figures 5B–5D, right, yellow versus gray boxes; Figures S7B and S7C, yellow versus gray boxes), as expected.

Next, we used qPCR to more quantitatively assess 12 control sites that lacked FoxA2 binding: 6 sites at non-accessible nucleosomes, but within 2 kb proximal to FoxA2-bound accessible nucleosomes (e.g., Figure S6A), and 6 sites at accessible nucleosomes not bound by FoxA2 (e.g., Figure S6B). Most control sites did not change their MNase accessibility in the FoxA1/A2 mutants (Figures S6D and S6E). Thus, the FoxA factors do not affect nucleosome accessibility nonspecifically at sites where they do not bind. By contrast, all 12 accessible nucleosomes with FoxA2 binding events at enhancers (e.g., Figure S6C)

Figure 5. FoxA2 Binding Is Required to Keep Nucleosomes Accessible in Chromatin

(A–E) Heatmap of (A) FoxA2 ChIP-exo in wild-type, (B) low-MNase-seq, (C) high-MNase-seq, (D) low-over high-MNase-seq, and (E) H1 ChIP-seq in wild-type (WT) and the FoxA1/A2 deletion mutant (Δ) at liver-specific enhancers, rank ordered by FoxA2 ChIP-exo read density. Box and whisker plots show at the central 200 or 1,000 bp of the top-25% FoxA2-occupied (FoxA2-strong) enhancers and at the bottom-25% FoxA2-occupied (FoxA2-weak) enhancers. p values by Wilcoxon rank-sum test. See also Figures S5–S7.



had significantly decreased MNase accessibility in the mutants (Figure S6F). Furthermore, 7 sites have their nearest gene exhibiting diminished expression of more than 2-fold in FoxA mutants (Li et al., 2011) (Figure S6G), associating FoxA binding with gene activation. Taken together, low- and high-MNase digestions reveal that the pioneer factors FoxA1 and FoxA2 help maintain an accessible nucleosome configuration at liver-specific enhancers in vivo.

FoxA Binding Displaces Linker Histone H1 from Nucleosomes

Interestingly, the “winged helix” DNA-binding domain structure of FoxA proteins highly resembles that of linker histone H1 (Clark et al., 1993; Ramakrishnan et al., 1993), the latter of which binds near the dyad axis of the nucleosome (Goytisolo et al., 1996), as does FoxA2 (Figure 3E). A single-locus study showed that induction of FoxA caused reduction of H1 occupancy at *Alb1* enhancer (Taube et al., 2010), similar to what had been reported from in vitro

studies with purified proteins (Cirillo et al., 2002; Cirillo et al., 1998). Here, we assessed the genome-wide occupancy of linker histone H1 in mouse hepatocytes of wild-type controls and FoxA1/A2 deletion mutants (Figure S5B). First, in the wild-type, we found that higher FoxA2 occupancy correlates with H1 depletion (Figure 5E for ± 500 bp; p value = 2.1×10^{-6} ; Figure S7E for ± 100 bp; p value = 5.5×10^{-11} ; compare yellow boxes at FoxA2-strong versus FoxA2-weak), suggesting that FoxA binding is involved in displacement of linker histone. In the deletion mutants, enhancers showed a striking increase in H1 deposition (p value < 2.2×10^{-16}) (Figure 5E for ± 500 bp; Figure S7E for ± 100 bp; yellow versus gray boxes). In contrast, silent heart enhancers, where FoxA2 was not bound, did not show an effect of FoxA deletion on H1 deposition level (Figure S7D). These in vivo genetic results indicate that FoxA binding displaces linker histones from the local chromatin, providing

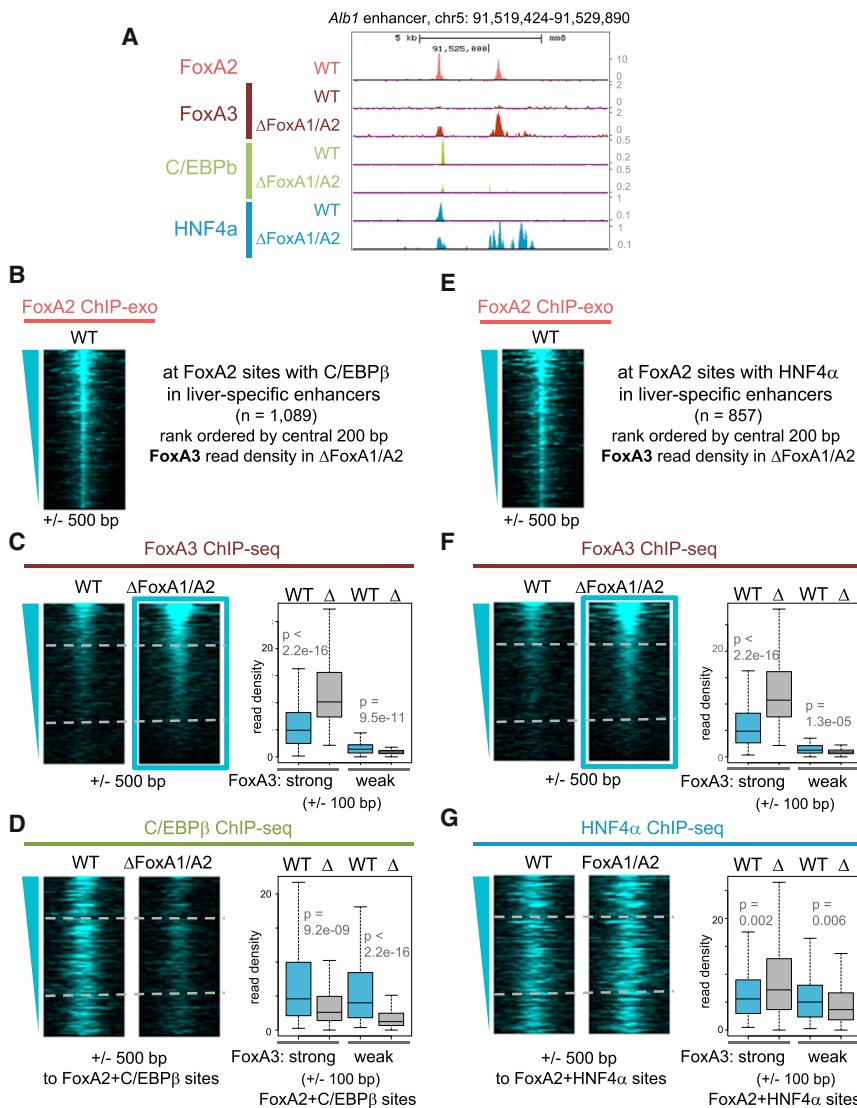


Figure 6. FoxA Binding Allows Other Liver-Enriched TFs to Bind

(A) FoxA2 ChIP-exo in wild-type and FoxA3, C/EBPβ, and HNF4α ChIP-seq signal tracks in wild-type and the FoxA1/FoxA2 deletion mutant.

(B–D) Heatmaps of FoxA2, FoxA3, and C/EBPβ ChIP-seq in wild-type (WT) and the FoxA1/A2 mutant (Δ) at FoxA2 binding sites overlapping with the C/EBPβ binding site in liver-specific enhancers, rank ordered by FoxA3 ChIP-seq signal in the FoxA1/A2 mutant.

(E–G) Heatmaps of FoxA2, FoxA3, and HNF4α ChIP-seq in wild-type and the FoxA1/A2 mutant at FoxA2 binding sites overlapping with HNF4α binding site in liver-specific enhancers, rank ordered by FoxA3 ChIP-seq read density in the FoxA1/A2 mutant. Box and whisker plots show at central 200 bp of the top-25% FoxA3-occupied (FoxA3-strong) enhancers and at the bottom-25% FoxA3-occupied (FoxA3-weak) enhancers. p values by Wilcoxon rank-sum test. See also Figure S6.

FoxA3 level in the mutants (Figure S5B). Around the *Alb1* enhancer, there were two FoxA2 binding peaks but no FoxA3 signal in the wild-type (Figure 6A). However, in the FoxA1/A2 mutants, we found modest emergence of FoxA3 signal at a 5' site, where C/EBPβ binding was diminished and HNF4α binding was slightly decreased, and a strong emergence of FoxA3 signal at the 3' site, where de novo HNF4α binding occurred (Figure 6A). These findings reveal partial compensation by FoxA3.

Next, we analyzed the genome-wide effects of FoxA1/A2 deletion at those FoxA2 binding sites that overlapped with C/EBPβ binding (Figures 6B–6D) or with HNF4α binding (Figures 6E–6G)

a simple explanation for the underlying nucleosomes becoming more accessible.

FoxA Binding Helps Other Transcription Factors Bind to Their Target Sites

Earlier studies indicated that binding of TFs, such as CREB, GR (glucocorticoid receptor), and ER (estrogen receptor), are dependent on pioneer factor FoxA binding (Carroll et al., 2005; Li et al., 2012; Zhang et al., 2005). Here, we examined the genome-wide consequences of FoxA1/A2 deletion on C/EBPβ and HNF4α binding, using the FoxA1/A2 deletion mutants (Figure S5B). We also performed ChIP-seq for FoxA3, the third of the three FoxAs expressed in liver, in order to define compensatory effects in the FoxA1/A2 deletion background. Microarray results showed that C/EBPβ, HNF4α, and FoxA3 expression were not changed beyond 2-fold in the FoxA1/A2 deletion mutants (Li et al., 2011), and western blot analysis showed a slight reduction of HNF4α level and no difference of C/EBPβ and

at liver-specific enhancers. We rank ordered the sites by FoxA3 ChIP-seq read density in the FoxA1/A2 mutants and curated the top 25% of sites (FoxA3-strong) where FoxA3 binding was significantly increased and could compensate FoxA1/A2 loss in the mutants, and the bottom 25% sites (FoxA3-weak), where FoxA3 compensation was minimal (Figures 6C and 6F). We found a striking reduction of C/EBPβ binding at FoxA3-weak sites (Figure 6D, right blue versus gray boxes) and FoxA3-strong sites (Figure 6D, left blue versus gray boxes) in the FoxA1/A2 mutants, indicating that FoxA1 and FoxA2 are required for C/EBPβ recruitment to their target sites.

As was seen at the *Alb1* enhancer sites, genome-wide HNF4α binding showed an increase upon FoxA1/A2 deletion at FoxA3-strong sites (Figure 6G, left blue versus gray boxes), but not at FoxA3-weak sites (Figure 6G, right blue versus gray boxes), suggesting that FoxA3 recruits HNF4α more efficiently than FoxA1/A2. We conclude that the FoxA family of pioneer factors

enhances the binding of different liver-specific transcription factors to liver enhancers.

Maintenance of Accessible Nucleosomes by FoxA for Liver-Specific Gene Activation

We assessed how the maintenance of nucleosome accessibility by FoxA relates to the regulation of target genes. We curated published gene expression profiles of the liver (Li et al., 2011) into upregulated genes and downregulated genes in the FoxA1/A2 mutants. We found 199 genes that were at least 2-fold downregulated (Figure 7A) and 406 genes that were at least a 2-fold upregulated (Figure 7B; Table S3) in the FoxA1/A2 deletion mutants. Tissue expression analysis and KEGG pathway analysis (DAVID Bioinformatics Resources, UniProt tissue expression database; Huang da et al., 2009) indicated that the downregulated genes were related to liver function, including various metabolic pathways (Figure 7A; Table S3), as expected for FoxA targets, whereas the upregulated genes were related to non-liver lineages (e.g., mammary gland, spleen, and kidney) (Figure 7B; Table S3), consistent with previous studies showing that FoxA occupies certain sites that can be repressed in the adult liver (Watts et al., 2011). We found only a few FoxA2 binding sites proximal to TSSs (± 300 bp) at these target genes (Figures 7A and 7B), indicating that FoxA2 binding at the promoter has a minimal effect on the expression of these genes. We assessed the predicted enhancer-promoter pairs in liver (Shen et al., 2012) to find enhancers that linked to differentially expressed genes in the FoxA1/A2 deletion mutants. We found 618 enhancers associated with downregulated genes and 512 enhancers associated with upregulated genes in the deletion mutants (Figures 7A and 7B). We found 114 FoxA2 binding sites in the enhancers linked to downregulated genes and 81 FoxA2 binding sites in the enhancers linked to upregulated genes (Figures 7A and 7B).

Next, we focused on these FoxA2 target sites and analyzed how the nucleosomal state was changed in the FoxA1/A2 mutants by low- and high-MNase-seq. At FoxA2 binding sites linked to downregulated target genes, nucleosome accessibility was decreased significantly in the FoxA1/A2 deletion mutants (p value = 0.01) (Figure 7C), as shown by reduced low-MNase signals and increased high-MNase signals (Figure S7F). By contrast, at FoxA2 binding sites linked to upregulated target genes, nucleosome accessibility did not change significantly in the FoxA1/A2 deletion mutants (p value = 0.353) (Figures 7D and S7G).

We also analyzed how TF binding was affected at the FoxA2 binding sites linked to down- or upregulated target genes in the FoxA1/A2 deletion mutants. At the FoxA2 sites linked to downregulated genes, where FoxA3 binding did not increase significantly and was therefore unable to compensate for FoxA1/A2 loss, nucleosome accessibility decreased in the mutants and C/EBP β binding was significantly decreased, while HNF4 α binding was modestly decreased (Figures 7C and 7E). Thus, C/EBP β was more sensitive to the change in nucleosome accessibility than HNF4 α . In contrast, at the FoxA2 sites linked to upregulated genes, where FoxA3 binding was significantly increased and therefore could compensate for FoxA1/A2 loss, nucleosome accessibility did not change in the mu-

nants, and C/EBP β and HNF4 α binding did not change (Figures 7D and 7F). Thus, FoxA3 can, to some extent, compensate for FoxA1/A2 loss to maintain nucleosome accessibility. Furthermore, we performed de novo motif analysis around the FoxA2 binding sites (± 100 bp) linked to upregulated genes and revealed ER/Rfx motifs (E value = $3.8e-06$), Zfp/E2f6/Sp motifs (E value = $4.2e-03$), and an RAR (retinoic acid receptor) motif (E value = $1.2e-02$), each of which can function as a repressor, e.g., ER in the absence of ligand (Li et al., 2012). Altogether, FoxA binding is required to maintain an accessible nucleosome configuration and recruits other liver-enriched TFs at functional liver enhancers.

DISCUSSION

The distinct mechanistic features of tissue-specific versus ubiquitous regulatory sequences are critical to understand gene regulation (Zabidi et al., 2015), yet structural features that distinguish these classes have been elusive. Here, we find that liver-specific enhancers retain MNase-accessible nucleosomes more than active promoters and ubiquitous enhancers. Furthermore, the basis by which FoxA proteins, and possibly other pioneer factors, initially open chromatin at target sites, genome-wide, had been unclear (Iwafuchi-Doi and Zaret, 2014). Here, we find that FoxA1 and FoxA2 binding displaces linker histones, thereby helping to keep target nucleosomes accessible, which in turn helps recruit other liver enriched TFs to stimulate liver gene transcription (Figure 7G).

Linker histones normally promote a closed nucleosome configuration. The DNA-binding domain of the linker histone highly resembles that of FoxA (Clark et al., 1993; Ramakrishnan et al., 1993) and binds near the dyad axis of the nucleosome (Goytisolo et al., 1996), as we found for FoxA (Figure 3E). Therefore, the displacement of H1 can be accomplished by competition between FoxA and linker histone binding near the nucleosome dyad. Interestingly, we found that H1 is displaced not only at FoxA high-occupied liver enhancers but also at FoxA low-occupied liver enhancers. Previous studies had reported that a FoxA mutant that disrupts sequence-specific binding, yet still retains nonspecific DNA and nucleosome binding, can scan chromatin in cells (Sekiya et al., 2009) and remain bound to chromosomes in mitosis (Caravaca et al., 2013). Such scanning could model a first step to make nucleosomes accessible at both weak and strong binding sites, with a second, more specific step to create a stably accessible nucleosome at strong sites. Regardless, our findings suggest that it would be useful for those studying other pioneer factors in development, cell reprogramming, and cancer (Zaret and Carroll, 2011) to investigate the role of H1 and its displacement in activating target genes.

Although the cooperative binding of TFs can be based on their protein-protein interactions, nucleosomes can facilitate cooperative binding without the direct factors' interactions; referred to as nucleosome-mediated cooperativity between TFs (Chávez and Beato, 1997). For example, nucleosome depletion at the mouse mammary tumor virus (MMTV) promoter leads to better accessibility for individual TFs, such as NFI (nuclear factor I) and GR, whereas the functional synergism between these TFs, which is required for strong hormonal induction, is only

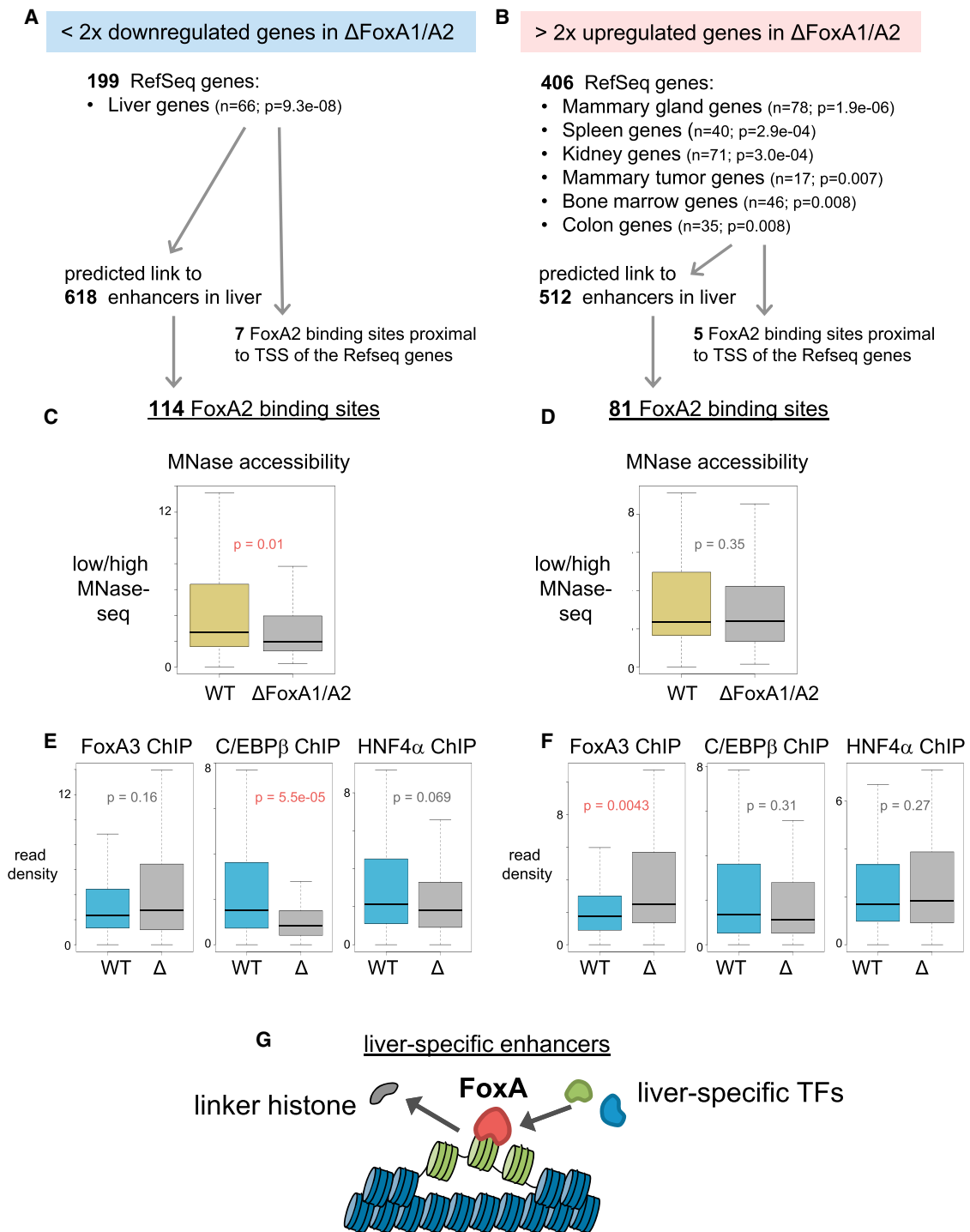


Figure 7. Maintenance of Accessible Nucleosomes by FoxA Is Required for Liver-Specific Gene Activation

(A and B) Tissue Expression analysis for >2-fold upregulated or downregulated genes in the FoxA1/A2 deletion mutant (Li et al., 2011; Table S3). Number of enhancers that potentially link to the target genes (Shen et al., 2012) and number of FoxA2 ChIP-exo sites in the enhancers.

(C and D) Box and whiskers plot shows MNase accessibility (low/high MNase-seq) in wild-type and the FoxA1/A2 mutant at the central 200 bp of downregulated genes related to FoxA2 sites and (D) at upregulated genes related to FoxA2 sites (Figure S7).

(E and F) Box and whiskers plot shows FoxA3, C/EBP β , and HNF4 α ChIP-seq in wild-type (WT) and the FoxA1/A2 mutant (Δ) at the central 200 bp of down-regulated genes related to FoxA2 sites (E) and at upregulated genes related to FoxA2 sites (F). p values by Wilcoxon rank-sum test.

(G) Models of nucleosome configuration at liver-specific enhancers. FoxA2 binding is required to displace linker histones, keep nucleosome accessible, and help recruitment of other liver enriched TFs, which stimulate liver gene transcription.

observed on nucleosomal DNA (Chávez and Beato, 1997). Our finding that the target genes of liver-specific enhancers exhibited higher expression levels than those of ubiquitous enhancers, even though the liver-specific enhancers have a low histone modification state, agrees with what has been observed at developmentally regulated genes in vivo (Pérez-Lluch et al., 2015). Taken together, it indicates that the accessible nucleosomes can participate actively in regulatory processes by facilitating cooperativity between TFs and consequent strong liver gene induction. In contrast, ubiquitous enhancers retained fewer nucleosomes and were mainly enriched for ubiquitous TF motifs, some of which (e.g., Sp1) are known to recruit chromatin-remodeling factors. The major structural differences between liver-specific and ubiquitous enhancers therefore associate with the different kinds of gene regulation.

Computational studies on mammalian genomes show that clusters of TF binding sites at enhancers are enriched for DNA sequences that encode higher intrinsic nucleosome occupancy compared to flanking sequences (Gaffney et al., 2012; Lidor Nili et al., 2010; Tillo et al., 2010). Furthermore, other TFs such as Sox2, Oct3/4, Klf4, p53, PU.1 (Spi1), and PR (progesterone receptor) can target binding sites with high nucleosome occupancy (Ballaré et al., 2013; Barozzi et al., 2014; Lidor Nili et al., 2010; Soufi et al., 2015). Most of them are also known as pioneer factors, which can allow other TFs to access their target sites (Iwafuchi-Doi and Zaret, 2014). Given that pioneer factors on their own can bind nucleosomal DNA and create accessible nucleosomes, we suggest that pioneer factors can initiate nucleosome-mediated cooperativity between TFs for strong tissue-specific gene activation.

MNase-qPCR has been applied with 16 levels of MNase digestion and the titration curve predicted the fraction of nucleosome occupancy in a cell population (Floer et al., 2010). While this is a powerful quantitative method to analyze a single target locus, it has not yet been scaled to the genome-wide level. Our genome-wide method, comparing low and high MNase-seq coupled with histone signals, was sufficient to reveal specific features of ubiquitous versus tissue-specific gene regulatory sequences in open chromatin that were previously thought to be nucleosome-free, the latter due to an experimental bias in high-MNase-seq nucleosome mapping. Our method provides a more accurate view of how transcription factors bind and function in chromatin and led to the unexpected insight that tissue-specific and ubiquitous enhancers exhibit structural differences. Given that transcriptional enhancers are increasingly being appreciated as contributing markedly to human health and disease (Kasowski et al., 2013), it is critical to obtain a better understanding of their structure and mechanism of action.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for more information.

MNase Treatment of Nuclei and Mono-nucleosomal DNA Extraction

Animal use was approved by an institutional animal care and use committee. Mouse liver nuclei were suspended in RSB (10 mM Tris [pH7.4], 10 mM NaCl, 3 mM MgCl₂, 10 mM sodium butyrate, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mg/ml pepstatin, and 3 mM CaCl₂), and the nuclear suspension was pre-warmed for 1.5 min at 37°C and treated with 0.5 or 1 U/ml MNase (Worthington

Biochemicals) for low digestion and 20 U/ml MNase for high digestion for 2 min at 37°C. After purifying DNA, mono-nucleosomal DNA (~140–200 bp) was extracted.

MNase-Seq

Two biological replicates of MNase-seq libraries were sequenced as 100-bp paired-end fragments, and MNase-seq libraries from FoxA1/FoxA2-flox; AAV-Cre mutant and WT; AAV-Cre were sequenced as 37-bp paired-end fragments. The sequence tags were aligned using Bowtie v0.12.5 (parameters -m 1, -best, paired-end alignment) to assembly NCBI v36 of the mouse genome (mm8).

ACCESSION NUMBERS

The accession number for the data reported in this paper is GEO: GSE57559.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found online with this article at <http://dx.doi.org/10.1016/j.molcel.2016.03.001>.

AUTHOR CONTRIBUTIONS

Conceptualization, M.I.-D. and K.S.Z.; Investigation, M.I.-D.; Bioinformatics, G.D., M.I.-D., A.K., S.M., and B.F.P.; Resources, J.W., D.L., and K.H.K.; Writing, M.I.-D. and K.S.Z.; Funding Acquisition, K.S.Z., K.H.K., B.F.P.; Supervision, K.S.Z.

ACKNOWLEDGMENTS

We thank M. Teta-Bissett and T. Bernard-Banks for FoxA mutant mice; B. Ren and F. Yue for enhancer data; B. Hoffman and P. Hoodless for HNF4a ChIP-seq data; K.Y. Chan and S.K.H. Han for ChIP-exo preparation; N. Bramswig, S. Shin, and P. Sen for experimental advice; M. Lazar, A. Soufi, and J. Kim for comments; and E. Hulme for manuscript preparation. M.I.-D. was supported by postdoctoral fellowships from JSPS and the Naito, Astellas, and Uehara foundations. B.F.P. has a financial interest in Peconic, LLC, which utilizes the ChIP-exo technology implemented in this study and could potentially benefit from the outcome of this research. This research was supported by NIH grants R37GM36477 (to K.S.Z.), P01DK049210 (to K.H.K.), and HG004160 (to B.F.P.).

Received: August 12, 2015

Revised: February 5, 2016

Accepted: February 29, 2016

Published: April 7, 2016

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